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THE EFFECTS OF LOW LEVEL PRENATAL CARBON MONOXIDE ON NEOCORTICAL DEVELOPMENT

by

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Abstract

The prevalence of maternal smoking remains surprisingly high, ranging from 10-50% of pregnant women across different countries and socioeconomic groups. This is associated with several adverse neurological outcomes including increased risk for Sudden Infant Death Syndrome, attention deficit disorder, and autism. Prenatal exposure to carbon monoxide (CO), a major toxic constituent of cigarette smoke, disrupts development of diverse brain structures and associates with similar neurological disorders. While the global effects of CO on the developing brain have been studied, the specific effects of CO on neocortical development and neuronal migration have not. In the first set of experiments we determined if CO exposure resulted in structural changes of the neocortex. Pregnant mice (CD1) were exposed to different concentrations of CO approximating levels found in smokers (0 or 150 ppm) from embryonic day 7 (E7) until birth. Bromodeoxyuridine (BrdU) was injected on either E12 or E16 to map the distribution of neocortical cells born on that date. Neocortical cells born early during corticogenesis (E12) were abnormally distributed toward the upper layers

while later born neocortical cells (E16) were abnormally distributed toward the middle layers of the neocortex in adult mice exposed to CO *in utero*. A greater percentage of BrdU⁺ cells colocalized with GAD 65/67 in treated vs controls, suggesting impairment of interneuron migration. Furthermore, the displaced cells did not co-localize with markers for projection neurons including MAP2 and CAMK-II. Treated mice also had altered distributions of GABAergic interneurons throughout the neocortex with a decreased density in the upper layers and an increased proportion of cells in central layers, suggesting they did not reach their intended target. Interneuron subtypes were differentially affected with a decreased distribution of parvalbumin immunoreactive neurons in the central layers and an increased proportion of calretinin immunoreactive neurons in the upper layers. This led us to question if the disordered cerebral cortex after CO exposure *in utero* produced functional changes in adult mice. In behavioral tests, exposed mice behaved similarly to controls in the open field test. However, CO exposed mice failed to demonstrate prepulse inhibition to the acoustic startle test, suggesting impairment of sensorimotor gating. CO exposed mice also had significant hyperalgesia compared to controls, suggesting lack of suprasegmental control. The final set of experiments aimed to understand the mechanisms underlying the displaced neurons by studying the direct effects of CO on migrating neurons during cortical development. Mice exposed to CO *in utero* had decreased levels of cGMP and phosphorylated VASP at birth, suggesting the NO-cGMP-cGK pathway may be impaired. To further investigate this mechanism, organotypic brain slices were grown in both normal and CO (50 ppm) environments. The ganglionic eminence was labeled with the fluorescent dye Dil. After 3 days in culture, the slices were fixed and analyzed. Treatment

with CO resulted in a reduction in the percentage of cells reaching the cortical plate. The CO-exposed cells maintained proper orientation, but had significantly shorter leading processes compared to controls. When co-treated with the phosphodiesterase inhibitor IBMX, CO treated slices showed improved migration and recovery in the length of the leading processes in migrating cells. These data provide evidence that CO impairs migration of interneurons by affecting important cytoskeletal proteins. This effect can be explained by CO acting as a partial agonist at soluble guanylate cyclase, thereby decreasing the formation of cGMP and decreasing the phosphorylation of VASP, its downstream target. In conclusion, these results indicate that CO contributes to the etiology underlying the neurological consequences of maternal smoking by impairing the migration of interneurons into the developing neocortex and altering sensory processing in the cerebral cortex.

**THE EFFECTS OF LOW LEVEL PRENATAL CARBON MONOXIDE ON
NEOCORTICAL DEVELOPMENT**

By

2nd Lt John F. Trentini III

United States Air Force

Doctoral Dissertation submitted to the faculty of the Graduate Program in
Neuroscience at the Uniformed Services University in partial fulfillment of the
requirements for the degree of Doctor of Philosophy, 2010.

DEDICATION

I dedicate this work to my parents, John and Rose Trentini, who didn't worry about how my neurons got to the correct layer of the cortex, but made sure I used every single one to its maximum potential.



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List of Abbreviations

ACSF	Artificial cerebrospinal fluid
ADHD	Attention Deficit/Hyperactivity Disorder
ASR	Acoustic startle reflex
BrdU	Bromodeoxyuridine
CGE	Caudal ganglionic eminence
cGK	cGMP-dependent kinase
cGMP	Cyclic guanosine mono-phosphate
CR	Calretinin
CO	Carbon Monoxide
Dil	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
HbCO	Carboxyhemoglobin
HO	Heme oxygenase
IBMX	3-isobutyl-1-methylxanthine
IZ	Intermediate zone
GAD	Glutamic acid decarboxylase
GE	Ganglionic eminence

MAM	Methyazoxymethanol
MAP2	Microtubule associated protein-2
MAPK	Mitogen-activated protein kinase
MGE	Medial ganglionic eminence
NO	Nitric oxide
PPI	Pre-pulse inhibition
PV	Parvalbumin
SIDS	Sudden Infant Death Syndrome
VASP	Vasodialator stimulated phosphoprotein
VZ	Ventricular zone

Chapter 1

INTRODUCTION

The prevalence of maternal smoking remains surprisingly high, ranging from 10-50% of pregnant women across different countries and socioeconomic groups (Toro *et al.*, 2008). In the US, 12-24% of pregnant women continue to smoke throughout pregnancy (Phares *et al.*, 2004). Prenatal exposure to cigarette smoke associates with a number of adverse clinical effects such as decreased birth weight (Ward *et al.*, 2007) and decreased head circumference at birth (Kallen, 2000), as well as adverse neurological outcomes including increased risk for Sudden Infant Death Syndrome (SIDS) (Pinho *et al.*, 2008), attention deficit/hyperactivity disorder (ADHD) (Milberger *et al.*, 1998) and autism (Hultman *et al.*, 2002). Maternal smoking also has long term behavioral consequences in offspring that are often subtle and overlooked clinically. Children born to women that smoke during pregnancy exhibit poorer performance on cognitive tasks and language development (Gusella and Fried, 1984, Fried and Watkinson, 1988, 1990). Fried *et al.* also observed that children born to heavy smokers (>20 cigarettes/day) compared to non-smokers had differences in a number of behavioral domains including auditory processing, attention, and language comprehension (Fried, 1995). A recent retrospective cohort study of 314 adolescents showed that those exposed to maternal cigarette smoke during gestation had significantly thinner cerebral cortices in brain regions associated with cognition, memory and emotional processing compared to non-exposed (Toro *et al.*, 2008). The consequences of maternal smoking in humans have been described epidemiologically, and the mechanisms mediating these effects have been shown in animal models.

The effects of maternal smoking are reported in a number of species. In primates, perinatal exposure to environmental tobacco smoke results in upregulation of nicotinic acetylcholine receptors throughout the brain, indicative that developing cholinergic activity may be impaired (Slotkin *et al.*, 2002). In adolescent rats, *in utero* exposure to cigarette smoke produced decreased arousal levels and impaired sensory gating (Garcia-Rill *et al.*, 2007). Good *et al.* showed in rats that exposure to maternal smoking resulted in hyperexcitability of neurons in the pedunculo pontine nucleus in the offspring (Good *et al.*, 2006). This region is part of the cholinergic arm of the reticular activating system, and modulates attention and alertness. Dysfunctional activity of this region is linked to ADHD, which reinforces the association linking maternal smoking with attention disorders (Konrad *et al.*, 2006). Cigarette smoke contains hundreds of toxic chemicals including acetone, arsenic, tar formaldehyde, and others (Stedman, 1968). The negative effects of maternal smoking are generally attributed to two major components of cigarette smoke: nicotine and carbon monoxide. While an abundance of research has demonstrated the biological consequences of maternal smoking, the vast majority of studies focuses primarily on the effects of nicotine, and often overlooks the effects of CO.

The effects of nicotine on the fetus result in hypoxic-ischemia due to constriction of the uterine arteries decreasing blood flow to the placenta, and also cause altered maturation of cholinergic circuits in the developing brain (Dwyer *et al.*, 2008). While many developmental effects have been attributed to nicotine, the effects of CO are often overlooked. The major effect of CO attributes to its ability to produce hypoxia by binding to hemoglobin forming carboxyhemoglobin and preventing O₂ delivery to tissues. CO readily crosses the placenta and binds

with fetal hemoglobin with a higher affinity than adult hemoglobin (Longo, 1970). CO can also bind other important heme containing molecules such as mitochondrial cytochromes of the electron transport chain, interfering with important energy generating processes (Chance *et al.*, 1970). A third mechanism of CO activity is as a gaseous neurotransmitter, similar to nitric oxide (NO), initiating a variety of intracellular cascades (Mann and Motterlini, 2007). Exposure to CO *in utero* has been shown to have many profound developmental effects in the developing brain, specifically the basal ganglia, cerebellum, hippocampus, cerebral cortex, and developing white matter tracts (Ginsberg and Myers, 1976, Daughtrey and Norton, 1982, Mereu *et al.*, 2000, Weiss *et al.*, 2004, Benagiano *et al.*, 2005). However at lower doses many of these effects can be subtle and often overlooked clinically; therefore this area of research remains a significant gap in the scientific and clinical literature. Furthermore, few have examined the direct effects of CO as a signaling molecule and its potential role in neuronal migration, a critical component of neocortical development. Therefore the objective of this research was to investigate the effects of prenatal CO on the developing brain, specifically to further understand the direct effects of CO on migratory cells in the developing cerebral cortex.

Neocortical development

Cortical development is a precisely regulated and complex process involving the proliferation, migration and differentiation of neurons. During embryology, the majority of neural progenitor cells arise from a neuroepithelial cellular region lining the ventricles called the ventricular zone (Rakic, 1982). During neurogenesis, most progenitor cells undergo symmetric cell division

producing two daughter cells of progenitor phenotype. As development continues, cells begin to divide asymmetrically, with a parent cell maintaining the progenitor fate and a daughter cell differentiating into a neuron (Caviness *et al.*, 1995). The cortex then develops in successive waves of cellular migration in two main patterns. Radial migration occurs when projection neurons migrate along radial glia from the ventricular zone following the radial orientation of the neural tube. In contrast, interneurons migrate tangentially from the ventricular zone of the ganglionic eminences of the ventral telencephalon initially roughly perpendicular to the direction of radial migration (Marin and Rubenstein, 2003). The timing of these migration patterns is well characterized through cell lineage and cell fate experiments in a variety of mammals, including humans, ferrets and rodents (Hatten, 1999, Marin and Rubenstein, 2003, Kriegstein, 2005, Ayala *et al.*, 2007). This process is visualized in a modified figure from a review article by Ayala and colleagues in 2007 (Figure 1).

In the mouse, around embryonic day 11 (E11), the first wave of neurons migrates radially to form the preplate. This layer is then split by the arrival of subsequent neocortical cells to form the marginal zone, which includes Cajal-Retzius cells and a deeper subplate layer containing the earliest generated cells (Marin-Padilla, 1998). The cortex then develops in an inside-out pattern as waves of cortical cells continue to insinuate between these two layers. The deeper layers of 6 & 5 form first followed by upper layers of 4 through 2 (Rakic, 1972, Bayer *et al.*, 1991). In mice and mammals in general, the timing of this process is well characterized in that the time a cell becomes post-mitotic predicts its target layer to which it will migrate. Inhibitory interneurons migrating tangentially rely on a number of intrinsic and extrinsic signals that stimulate

motility and guide the cell to its location. Generally interneurons migrate to the same layers according to their birthdate as the radially migrating cells. The tangentially migrating neurons are particularly vulnerable to insult, and perturbation of migration has profound effects on the structure and function of the cerebral cortex. In a ferret model of cortical dysplasia, our laboratory has shown that interruption of mitosis at E33, a critical period of corticogenesis, using methyazoxymethanol (MAM) alters thalamocortical projections, distribution of GABA_{Aα} receptors, GABAergic neurons and neuronal responses recorded through cortical layers (Noctor *et al.*, 1997, Noctor *et al.*, 2001, Palmer *et al.*, 2001, Jablonska *et al.*, 2004, McLaughlin and Juliano, 2005). This affect was attributed in part to aberrant migration patterns of tangentially migrating cells caused by MAM treatment, and is an example of an effective model of environmentally inducing a neuronal migration disorder (Poluch *et al.*, 2008). A number of other cortical migration disorders are characterized by abnormal migration and positioning of GABAergic interneurons (Wonders and Anderson, 2006).

Neuronal migration disorders occur when either genetic or environmental factors affect this process and result in cortical dysplasia. Cortical dysplasia is characterized by altered cellular morphology, ectopic cell clustering, and laminar disruption (Taylor *et al.*, 1971, Tassi *et al.*, 2002). Mutations in genes such as *LIS1*, *DCX*, *RLN*, and *ARX* result in cortical malformations including lissencephaly, doublecortex, and ectopic neuronal clusters (Gleeson *et al.*, 1999). Often cortical dysplasias are not associated with specific genes, and result from environmental factors. Exposure to environmental toxins such as cigarette smoke, alcohol, methyl mercury, or radiation can result in a myriad of

cortical defects based on abnormal proliferation and migration, aberrant cell death patterns, or abnormal gliogenesis (Kriegstein, 1996, Krauss *et al.*, 2003, Pang *et al.*, 2008). The most common clinical presentations are mental retardation, intractable epilepsy, depression, schizophrenia and cognitive deficits (Guerrini and Filippi, 2005, Pang *et al.*, 2008). However subtle changes, such as alterations in interneuron distribution, may not be overtly apparent in behavioral changes. Epidemiological associations with prenatal insults and developmental disorders have been described. Krauss *et al.* found a significant association with microcephaly, a neurological disorder characterized by an individual with a smaller than normal head circumference, and maternal smoking (Krauss *et al.*, 2003). In fact, mothers that smoke have a 2 times greater risk of having an infant with microcephaly than nonsmokers (Van den Eeden *et al.*, 1990). These data indicate that toxic components of cigarette smoke, including CO, may be a significant cause mediating some neuronal migration disorders.

Effects of Carbon Monoxide

CO is an odorless, colorless, tasteless gas formed from incomplete combustion. Some common sources of CO include cigarette smoke, vehicle exhaust, and poorly ventilated charcoal cooking. The major toxic effect of CO is the production of anemic hypoxia through the binding of hemoglobin forming carboxyhemoglobin (HbCO). This shifts the O₂ dissociation curve to the left, decreasing O₂ delivery to the tissues (Longo, 1976). In normal nonsmoking pregnant women, HbCO levels have been reported to vary between 0.4 and 2% likely from endogenous production of CO through heme metabolism in the mother and fetus (Gemzell *et al.*, 1958, Haddon *et al.*, 1961, Linderholm and

Lundstrom, 1969, Longo, 1970). Smokers can have HbCO levels upwards of 5 to 10% depending on frequency and quantity of smoking (Benowitz and Jacob, 2000, Cronenberger *et al.*, 2008). CO also binds to fetal hemoglobin with a higher affinity than adult hemoglobin and readily crosses the placenta, increasing the vulnerability of the developing fetus to insult (Longo, 1970). Experimental exposure to low levels of CO is an established method to simulate CO exposure in smokers and produces similar HbCO levels.

CO can also have direct effects on tissue. As a gaseous signaling molecule, CO can freely diffuse across the cell membrane to reach equilibrium with the extracellular environment, and act on a variety of intracellular sites (Figure 2). At elevated intracellular levels, CO can bind to important heme containing molecules in the cell including mitochondrial cytochromes of the electron transport chain, cytochrome P450 enzymes of metabolism, myoglobin, neuroglobin, and cytoglobin (Caughey, 1970, Sawai *et al.*, 2005, Fago *et al.*, 2006). CO has also been shown to function as a gaseous neurotransmitter in the same class as NO, and has been studied predominantly in the cardiovascular and immune systems. CO modulates many pathways that include the mitogen-activated protein kinase (MAPK)-related pathways, suppression of cytokine production via the JNK pathway, and the calcium-sensitive potassium channels in the carotid bodies (Morse *et al.*, 2003, Williams *et al.*, 2004, Kim *et al.*, 2006). The signaling role for CO first emerged from observations in smooth muscle cells that CO can stimulate soluble guanylate cyclase (sGC) to produce cGMP (Furchgott and Jothianandan, 1991). cGMP is an important signaling molecule for a number of developmental processes, including formation of the neuromuscular junction (Wang *et al.*, 1995), modulation of gap junctions in the

neocortex (Rorig and Sutor, 1996), neural plate responsiveness to Sonic hedgehog signaling (Robertson *et al.*, 2001), and neuronal growth cone extension and responsiveness to semaphorin 3A (Song *et al.*, 1998, Van Wagenen and Rehder, 2001). cGMP also activates cGMP dependent kinases (cGK's), which phosphorylate a number of important cytoskeletal proteins involved in neuronal migration, such as the actin associated cytoskeletal complex Ena/VASP (Goh *et al.*, 2002). These processes are essential for brain development under normal conditions. Environmental exposure to CO can alter these events and have dramatic effects on the developing fetus.

Prenatal CO and brain development

Prenatal exposure to CO produces a number of neurological effects. Maternal exposure to low levels of CO has been shown to disrupt development of the medulla (Tolcos *et al.*, 2000), basal ganglia (Daughtrey and Norton, 1982), cerebral cortex (Ginsberg and Myers, 1974), and the cerebellar cortex (Benagiano *et al.*, 2005), as well as the development of important myelinating oligodendrocytes (Weiss *et al.*, 2004). In adult rats exposed to CO prenatally, there is a disruption in long-term potentiation in the hippocampus, suggesting long term deficiencies in learning and memory (Mereu *et al.*, 2000). At low concentrations (75-150 ppm) CO induced a variety of neurobehavioral abnormalities in rat offspring including behavioral activity levels through preweaning, delayed development of homing behavior and negative geotaxis, altered ontogeny of emotional responsiveness, and permanent cognitive deficits (Fechter and Annau, 1977, 1980, Mactutus and Fechter, 1984, 1985, Di Giovanni *et al.*, 1993). Prenatal CO exposure also affects a variety of neurotransmitters

and enzymes in the developing brain. Cagiano *et al.* demonstrated in male rats that low CO concentrations elicited changes in mesolimbic dopaminergic function and impaired sexual behavior (Cagiano *et al.*, 1998). Storm and Fechter found changes in catecholamines in the cerebellum and linked these changes with deficiencies in motor test performance and in assessments of learning and memory (Storm and Fechter, 1985). Storm *et al.* noted a decrease in total GABA content in cerebelli of 10 day old rats exposed to CO prenatally, suggesting GABAergic neurons may represent a subpopulation of neurons particularly vulnerable to CO toxicity (Storm *et al.*, 1986). Benagiano *et al.* later demonstrated layer specific decreases in GABA content in the cerebellum, as supporting evidence of motor impairments produced (Benagiano *et al.*, 2005). Prenatal CO exposure also causes decreased levels of the important NO- and CO-generating enzymes nitric oxide synthase (NOS) and heme oxygenase (HO) in the hippocampus (Vaccari *et al.*, 2001). While many toxic effects of CO on the brain have been described, none have examined the effects on the developing neocortex. Furthermore, most studies attribute the effects of CO toxicity to hypoxia induced by the formation of HbCO, and few have studied direct effects of increased CO on the developing brain.

CO and neuronal migration

Hypotheses on CO signaling arise from the evidence that CO participates in many of the NO-mediated signaling pathways. The role of NO-mediated production of cGMP through the activation of soluble guanylate cyclase in neuronal migration has been studied in insect models of development. The development of the insect enteric nervous system is a well-established model to

study neuronal migration (Hartenstein, 1997). In the hemimetabolous grasshopper (*Locusta migratoria*), Haase and Bicker showed that NO-induced production of cGMP is an essential component of neuronal migration. Neurons fated for the midgut plexus exhibited an increase in NO-inducible cGMP immunoreactivity during their migratory phase of development. Furthermore, pharmacological inhibition of endogenous NOS, sGC, and cGK each significantly decreased neuronal migration (Haase and Bicker, 2003). CO is produced by heme oxygenase (HO) during the metabolism of heme (Boehning *et al.*, 2003), and can signal similar to NO via the cGMP/cGK cascade. In the grasshopper embryo, the enteric neurons express HO while migrating to the midgut, suggesting CO may also play a critical role in migration (Bicker, 2007).

Other recent work has examined the role of NO-induced cGMP production in neuronal migration and development. Ding *et al.* described the developmental expression profile of soluble guanylate cyclase in the mouse, which parallels the period of neuronal migration in the developing brain (Ding *et al.*, 2005). Furthermore, Demyanenko *et al.* showed that mice with deletion of the gene for soluble guanylate cyclase (sGC) had abnormal neocortical development consistent with deficiencies in neuronal migration (Demyanenko *et al.*, 2005). Another experimental study used a model of fetal alcohol syndrome in mice, and found that the inhibition of neuronal migration by ethanol was reversed by either increasing cGMP levels or decreasing cAMP levels (Kumada *et al.*, 2006). In the mouse embryo, tangentially migrating neurons from the GE naturally produce cGMP earlier than neurons situated in the cortex, and also produce cGMP in response to exogenous NO treatment (Currie *et al.*, 2006). The evidence that cGMP is critical for neuronal migration suggests that alterations of cGMP levels,

such as that induced by exogenous CO poisoning, may dramatically affect neuronal migration and neocortical development. While many of the effects of CO as a signaling molecule have been described, no studies focus on the effects of migrating cells in the developing mammalian brain.

Figure 1. Neuronal migration and development of the neocortex. (Modified from Ayala *et al.*, 2007)

Development of the cerebral cortex requires the succinct proliferation, migration and differentiation of neurons. In the coronal section of embryonic brain on left, neocortical neurons arise from the neocortical ventricular zone (green) and the lateral or medial ganglionic eminences (LGE, MGE; purple). At right, projection neurons originate from the neocortical ventricular zone and migrate radially using a radial glial scaffold (a). Interneurons originate from the ganglionic eminences of the ventricular zone, and migrate tangentially into the cortical plate (b). Once in the cortical plate, this interneuron switches to radial migration (c). Both of these neurons respond to position cues (d) and will share the same laminar position based on their birthdate (e).

Figure 1. Neuronal migration and development of the neocortex. (Modified from Ayala *et al.*, 2007)

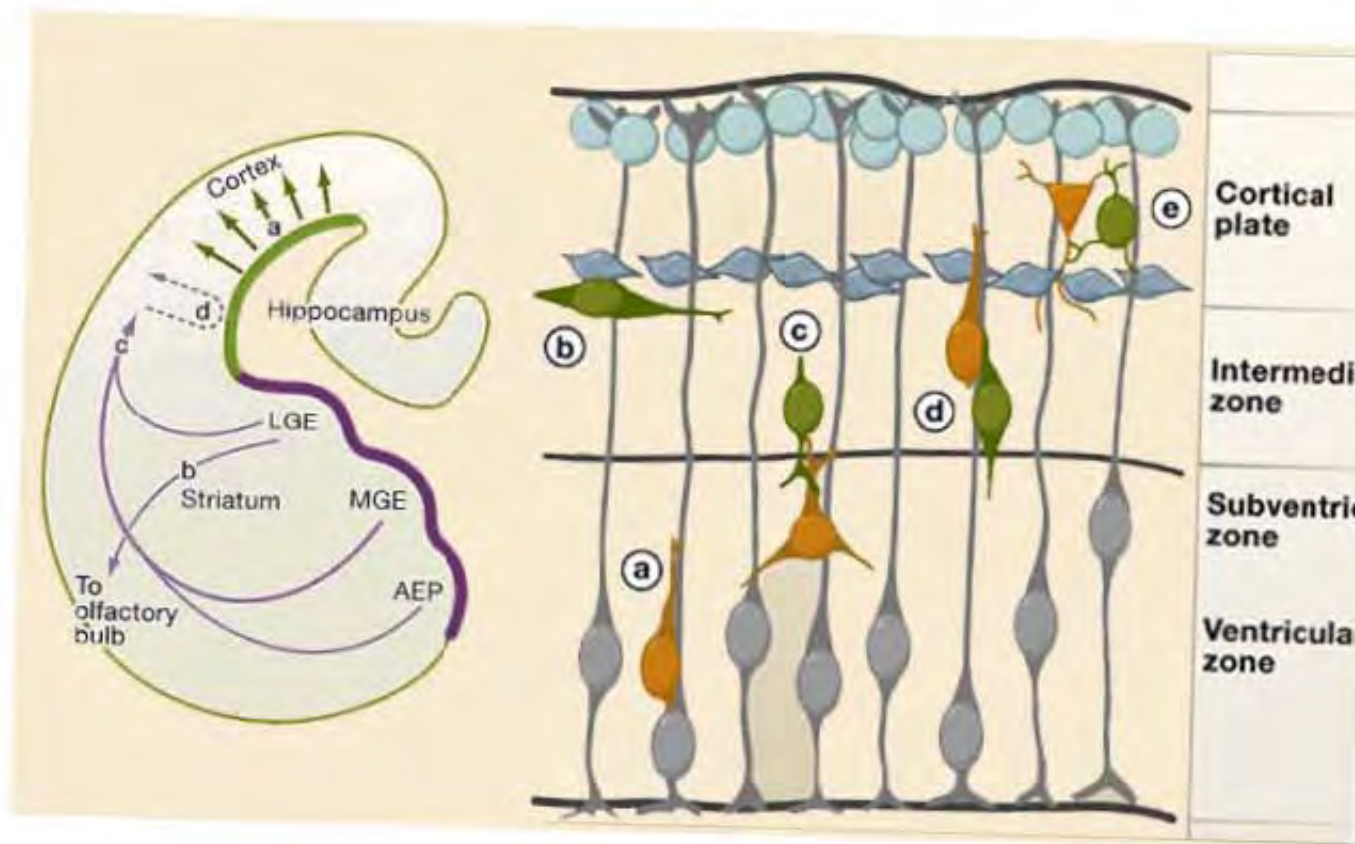
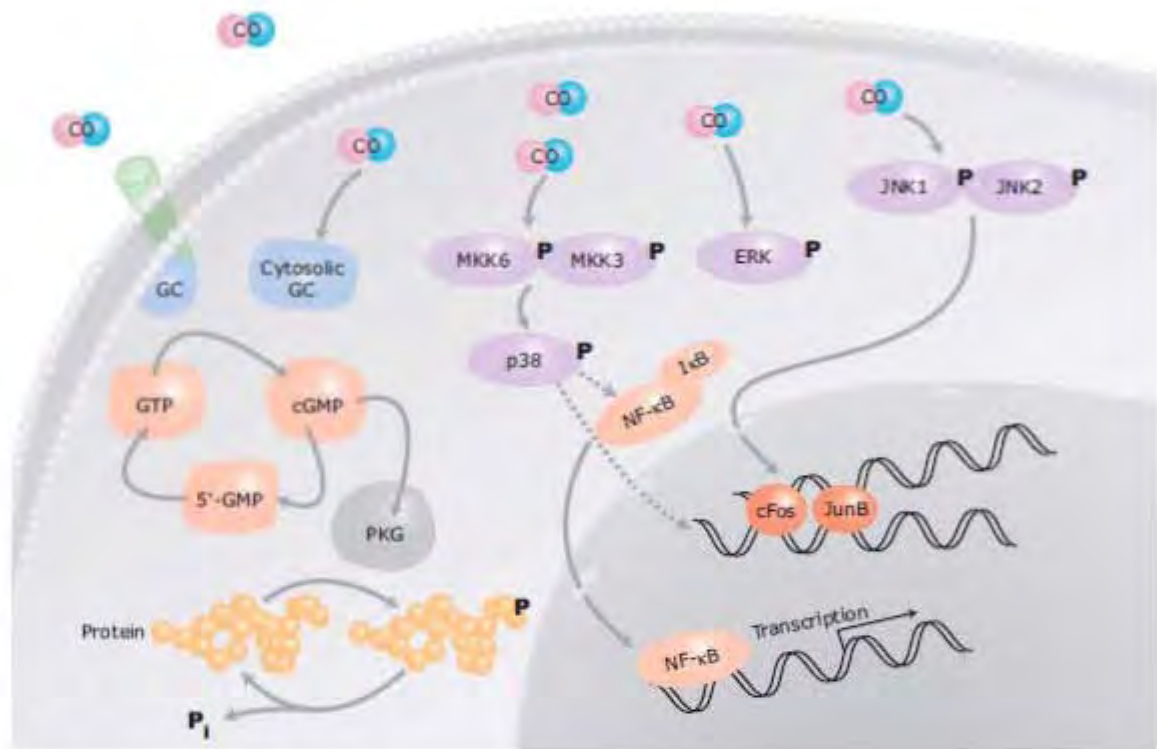


Figure 2. CO signaling pathways (from Ryter *et al.*, 2004)

CO affects a number of cellular signaling pathways illustrated in this diagram. CO can activate the heme moiety in soluble (cytosolic) guanylate cyclase, similar to NO, to produce cGMP. This results in stimulation of cGMP dependent kinases, which phosphorylate a number of effector proteins. CO can also modulate the MAPK, ERK, and JNK pathways, although the mechanism of action at these sites remains unclear.

Figure 2. CO signaling pathways (from Ryter *et al.*, 2004)



Chapter 2

OVERVIEW OF EXPERIMENTAL DESIGN

All animal work in this thesis was approved by the USUHS IACUC and used male and female CD-1 mice. For Specific Aims 1 and 2, time dated pregnant dams were obtained from a vendor (Charles River) and placed in environmental chambers from embryonic day 7 (E7)-birth. Mice were then removed from exposure chambers and returned to the colony. Male and female pups were examined postnatally as adults (P56 or later). For Specific Aim 3, embryos were removed from dated pregnant mice at E15 for organotypic slice culture experiments.

Specific Aim 1: Does prenatal CO exposure affect migration and laminar fate of neurons?

Despite evidence from a number of systems that CO signaling may influence neuronal migration, very few studies have investigated the role of CO in the development and migration of neurons in the cerebral cortex. This aim allowed us to determine the effect of exogenous CO on neurons fated for specific layers of the neocortex.

Experiment 1. To map the disposition of cells born early or late day during neocortical development (E12 or E16), pregnant mice were injected with bromodeoxyuridine (BrdU). E12 corresponds to the birth of the subplate and neurons of deeper layers 5 & 6. E16 corresponds to the birth of upper layers of 2 & 3. At least 4 pups from different litters from each treatment group were sampled. The distribution of BrdU immunoreactive cells was analyzed and compared in the somatosensory cortex from three brain slices per mouse.

Experiment 2. Does prenatal CO differentially affect subpopulations of neurons?

To further understand if CO influences the migration of a specific neuronal subpopulation, in addition to analyzing the distribution of BrdU⁺ neocortical cells, the distribution, number and morphology of interneurons and projection neurons was assessed using antibodies directed against GAD 65/67 or MAP2 in adult mice exposed to 0 or 150 ppm CO *in utero*. To more precisely determine the phenotype of the displaced GABA⁺ cells, different antibodies directed against the GABAergic subtypes expressing calretinin or parvalbumin were also used.

Specific Aim 2: What is the functional outcome in behavior of mice exposed to CO prenatally? To assess the behavioral outcomes, adult CD-1 mice exposed to 0 or 150 ppm CO *in utero* participated in a variety of behavioral tests.

Experiment 3: Does prenatal CO exposure affect overall locomotor activity?

Improper migration of neurons into the neocortex may produce motor deficits. The most standardized general measure of locomotor function is spontaneous activity in the open field. The general locomotion and activity of each mouse was assessed on various parameters including general locomotion as well as time spent in the outer vs. middle quadrants. These data detected any gross motor or activity deficits in treated mice compared to controls.

Experiment 4: Does prenatal CO affect sensorimotor gating? Neuronal migration disorders are commonly associated with functional deficits such as

deficiencies in sensorimotor gating. The prepulse inhibition test is effectively optimized for mice. The sensorimotor gating of the mouse was assessed by measuring the startle response to a strong auditory stimulus (110 dB tone) following a weak stimulus (68, 79, or 90 dB tone). The amount of startle inhibition was measured as a function of weight displacement.

Experiment 5: Does prenatal CO affect central pain processing? Altered interneuron distribution may affect the overall excitatory/inhibitory tone of the cerebral cortex, which may result in altered sensory processing. To test the mouse's nociceptive threshold, the hot plate responses were measured using an Omnitech Electronics Hot Plate Analgesiometer (Omnitech Electronics, Columbus, OH). Mice were placed on a 26 x 26 cm square platform maintained at 51°C. Hotplate latencies were measured as the time from placement on the heated surface until the animal raised and licked a rear paw.

Specific Aim 3: What is the direct effect of CO on tangential migration of cells?

Experiment 6. Does CO directly affect migration of cells from the ganglionic eminence (GE)? To answer this question, organotypic slice cultures from E15 mice incubated at 0 or 50 ppm CO for 3 days in culture. Dil was injected into the GE to label and track the migration of cells. After 3 days in culture, slices were removed from the incubators, fixed and imaged. A number of parameters were measured including the total number of cells, distribution, orientation, and length of leading processes.

Experiment 7. What is the mechanism underlying altered neuronal migration in the presence of CO? To answer this question, the methods from experiment 6 were repeated with the addition of drugs to the culture media to target specific reactions of the NO/CO-cGK-cGMP pathway. To determine if this pathway mediates abnormal migration after exposure to CO, we pharmacologically blocked the breakdown of cGMP using the phosphodiesterase inhibitor IBMX. We then analyzed slices for the distribution, orientation, and length of leading processes of migrating cells.

CHAPTER 3

Prenatal CO exposure results in abnormal migration of interneurons into the cerebral cortex

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Abstract

Maternal smoking is associated with several adverse neurological outcomes including increased risk for Sudden Infant Death Syndrome, attention deficit disorder and autism. Prenatal exposure to carbon monoxide (CO), a major toxic constituent of cigarette smoke, disrupts development of many brain structures and associates with similar neurological diseases. While information about the global effects of CO on the developing brain is available, the specific effects of CO on neocortical development and neuronal migration are not. To assess the effect of CO on the neocortex, pregnant dams were exposed to either 0 or 150 ppm CO from embryonic day 7 (E7) until birth. To assess the distribution of neurons generated on specific days, BrdU was injected at either E12 or E16. The distribution of BrdU⁺ cells was subsequently analyzed in somatosensory cortex of the treated and control animals as adults. Exposure to low levels of CO resulted in a significant number of misplaced cells towards middle layers of the neocortex. The majority of these cells colocalized with GAD 65/67 suggesting impairment of interneuron migration. Furthermore, these displaced cells did not co-localize with markers for projection neurons (MAP2). Interneuron subtypes were also affected, with altered distribution of parvalbumin⁺ and calretinin⁺ neurons. To determine the functional consequence of CO exposure, mice were assessed on a variety of behavioral measures. CO exposed mice possessed normal overall locomotor activity, but did not demonstrate pre-pulse inhibition to the acoustic startle test. Exposed mice also demonstrated hyperalgesia when tested with the hot plate test. These results suggest that CO impairs structural development of the cerebral cortex and produces functional deficits in offspring.

Keywords:

Carbon monoxide, neocortical development, neuronal migration, mouse, BrdU, interneuron, GABA, behavioral test

Introduction

The prevalence of maternal smoking remains surprisingly high, ranging from 10-50% of pregnant women across different countries and socioeconomic groups (Toro *et al.*, 2008), and one in five pregnant women in the US (Phares *et al.*, 2004). Prenatal exposure to cigarette smoke associates with a number of adverse clinical effects such as decreased birth weight (Ward *et al.*, 2007) and decreased head circumference at birth (Kallen, 2000), as well as adverse neurological outcomes including increased risk for Sudden Infant Death Syndrome (SIDS) (Pinho *et al.*, 2008), attention deficit disorder (Milberger *et al.*, 1998) and autism (Hultman *et al.*, 2002). The negative effects of maternal smoking are generally attributed to two major components of cigarette smoke: nicotine and carbon monoxide. While an abundance of research has demonstrated the biological consequences of maternal smoking, the vast majority of studies focuses primarily on the effects of nicotine, and often overlooks the effects of CO.

Carbon monoxide is a colorless, odorless, tasteless gas formed by incomplete combustion of organic fuels. Exposure to CO *in utero* causes many profound effects in the developing brain, specifically the basal ganglia, cerebellum, hippocampus, cerebral cortex, and developing white matter tracts (Ginsberg and Myers, 1976, Daughtrey and Norton, 1982, Mereu *et al.*, 2000, Weiss *et al.*, 2004, Benagiano *et al.*, 2005). These effects have been largely attributed to the ability of CO to produce hypoxia by binding with hemoglobin at low levels. Few have examined the direct effects of CO as a signaling molecule and its role in neuronal migration, a critical component of neocortical development. Therefore the objectives of this study were to examine the effects

of prenatal CO on the developing neocortex and to characterize the functional consequences of exposure.

Neocortical development is a precisely regulated and complex process involving the proliferation, migration and differentiation of neurons. When a neural progenitor cell becomes post-mitotic, it migrates to its target layer before differentiating into a mature neuron. In mice, and mammals in general, the timing of this process is well characterized in that the embryonic date a cell undergoes its last mitotic division predicts its intended layer (Rakic, 1974). In general, projection neurons originate from the neocortical ventricular zone and migrate radially using a scaffold (Rakic, 1971). Inhibitory interneurons originate from the ganglionic eminences and migrate tangentially relying on a number of intrinsic and extrinsic signals that stimulate motility and guide the cell to its location. Generally interneurons migrate to the same layers as the radially migrating cells according to their birthdate (Lavdas *et al.*, 1999). Exposure to environmental toxins such as cigarette smoke, cocaine (Crandall *et al.*, 2004), alcohol (Kumada *et al.*, 2007, Nakamura *et al.*, 2007), bisphenol (Nakamura *et al.*, 2007), methyl mercury (Choi *et al.*, 1978), or radiation can result in a myriad of cortical defects based on abnormal proliferation and migration, aberrant cell death patterns, or abnormal gliogenesis (Kriegstein, 1996, Krauss *et al.*, 2003, Pang *et al.*, 2008).

Although the effects of prenatal CO exposure have been studied in the brain, the specific effects on neocortical development and neuronal migration have not. Here we report that prenatal exposure to low levels of CO results in a significant increase in displaced cells born on E12 or E16. Furthermore, CO exposure causes alterations in the distribution of interneurons, but not projection neurons in the neocortex. Subpopulations of interneurons expressing

parvalbumin or calretinin were also susceptible to this insult. Furthermore, CO exposure results in behavioral changes in mice consistent with disrupted neocortical development.

Materials and Methods

Animals and Exposures

All animal work was approved by the Uniformed Services University Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1987). Timed pregnant mice (CD-1) were purchased from Charles River Laboratories (Wilmington, MA). Pregnant dams were placed in sealed acrylic environmental chambers from E7 until birth. A CO concentration of 150 ppm was maintained by mixing 7.5% CO with room air with the flow controlled by meters and monitored with a calibrated meter (Interscan RM14-500M or 50.0 M, Chatsworth, CA).

Histology and laminar measurements

After cutting in a coronal plane, 40 μm thick sections were mounted and processed for staining with cresyl violet as previously described (Gittins and Harrison, 2004). In brief, slices incubated in filtered 0.75% cresyl violet for 4 mins, went through a series of dehydration steps from 50% to 100% ethanol, followed by a 5 min wash in xylene., and were permanently mounted with coverslips. Images were taken using at 10x magnification (Zeiss; Thornwood, NY). The cortical thickness was measured as the distance from the pia to the white matter tracts, and thickness of layers 1, 2-4, 5 and 6 was measured using Axiovision software.

BrdU birthdating

For birth dating studies, pregnant dams exposed to 0 or 150 ppm CO were injected 3x i.p. at 45 minute intervals with bromodeoxyuridine (BrdU; 50 mg/kg, Sigma) diluted in sterile saline at E12 or E16. Pups were examined postnatally as adults (P56 or later). Mice were anesthetized using euthasol (50 mg/kg; Virbac Animal Health), perfused with 4% paraformaldehyde in phosphate buffer (pH-7.4), brains dissected and sectioned coronally, and BrdU immunoreactive cells visualized immunohistochemically.

Immunohistochemistry

After cutting in a coronal plane at 40 μ m thick on a vibrotome, sections were incubated overnight at 4°C in either rabbit IgG antibodies against GAD 65/67 (1:500, Sigma), MAP2 (1:500, Sigma), or calretinin (1:500, Sigma), or mouse IgG antibodies against parvalbumin diluted in buffer containing phosphate buffered saline (PBS), normal goat serum (NGS, 10%), and Triton X-100 (0.3%). The slices were then washed in PBS and incubated with appropriate secondary antibodies (Alexa Fluor 546-conjugated goat anti-rabbit or goat anti-mouse, 1:1000). Slices containing somatosensory cortex were then subsequently processed for BrdU immunohistochemistry as previously described (Poluch *et al.*, 2008). In brief, slices were incubated in ethanol (70%) for 10 mins, then placed in 2N HCl at 37°C for 1 hr, and rinsed in 0.1 M borate buffer, pH 8.5 for 20 min. After washing in PBS, slices were incubated overnight at 4°C in polyclonal rat IgG antibodies against BrdU (1:200; Accurate Chemical and Scientific Corps., Westbury, NY) in buffer containing PBS, NGS (10%), and Triton X-100 (0.3%).

Slices were then washed in PBS, and then incubated for 3 hrs at room temperature with Alexa Fluor 488-conjugated goat anti-rat IgG (1:500).

Quantitative Analysis of Distribution

To quantify the laminar distribution of double labeled cells, images of 500 μ m wide rectangles of somatosensory cortex approximately 2 mm lateral to the midline were selected and divided into 10 equal bins from the pia to the ventricular zone. The number of labeled cells in each bin was counted and plotted as a percent of the total number of labeled cells. Cells with dense staining of more than half the nucleus were considered BrdU-positive (Costa *et al.*, 2001).

Behavioral Tests

Adult mice exposed to 0 or 150 ppm CO *in utero* were subjected to a variety of behavioral tests during their active (dark) cycles. Locomotion was measured using an Omnitech Electronics Digiscan infrared photocell system. Mice were placed in a 40 x 40 x 30 cm clear Plexiglas arena and locomotion monitored for 1 hour. Data was automatically gathered and transmitted to a computer via an Omnitech Model DCM-I-BBU analyzer. The acoustic startle reflex and pre-pulse inhibition test was measured in a Med Associates Acoustic Response Test System (St. Albans, VT). The mouse's movement in response to acoustic stimuli was measured as a voltage change by a strain gauge inside the platform. Startle stimuli were 110 dB white noise bursts (20 msec) preceded 100 msec by 68, 79, or 90 dB 1kHz pure tones (pre-pulse) (Paylor and Crawley, 1997). The hot plate responses were measured using an Omnitech Electronics Hot Plate Analgesiometer (Omnitech Electronics, Columbus, OH). Mice were

placed on a 26 x 26 cm square platform maintained at 51°C. Hotplate latencies were measured as the time from placement on the heated surface until the animal raised and licked a rear paw.

Statistical analyses

For neuronal distributions and laminar measurements, data was analyzed using a 2-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedure (Holm-Sidak method). The Holm-Sidak test was chosen because it is more powerful than the frequently used Bonferonni test for multiple paired comparisons. For behavior tests, data was analyzed using a Student's t-test. We considered a level a statistical significance to be reached at $p < 0.05$.

Results

Prenatal CO results in a thinner cerebral wall at P4

To determine if prenatal CO exposure resulted in gross anatomical changes, we examined the structure of somatosensory cortex in pups exposed to CO *in utero* at three ages: postnatal day 4 (P4), 11 (P11), or adult (P56 or later). Figure 1 is an example of a cresyl violet stained section used for laminar measurements. At P4, CO treated mice had significantly thinner cerebral cortices compared to controls ($707.5 \pm 38.7 \mu\text{m}$ vs $772 \pm 20.1 \mu\text{m}$; $p < 0.05$). When we examined specific layers, there were no significant differences in the relative size of each layer compared to the total cortical thickness (Table 1). At P11, CO treated mice recovered, showing no significant difference in overall cortical thickness or in cortical layers compared to controls. Adult mice also had no significant differences in either cortical thickness or laminar proportion.

Prenatal CO altered BrdU distribution

To determine if prenatal exposure to CO altered the laminar fate of cells, we examined the distribution of both early (E12) and later born (E16) cells using BrdU (Figure 2). Four different mice were used at each injection time, ensuring that at least three different litters were sampled. For consistency, the region of the brain containing the somatosensory cortex was analyzed. As described in the Methods, a 500 μm wide region of cerebral cortex was divided into 10 equal bins, with bin 1 corresponding to the region closest to the pia and bin 10 closest to the white matter tracts (see Figure 2). The total number of BrdU+ cells were counted in this region and plotted as a fraction of the total found in each bin. A higher power view of immunoreactive cells can be seen in Figure 2, which also shows

an image of the distribution of BrdU+ cells in a 500 μ m slab of cortex. In normal mice, BrdU+ cells after injection at E12, were found in the bins closer to the white matter, and were predominantly located in bins 8-10, as would be predicted from prior studies (Price *et al.*, 1997, Yozu *et al.*, 2004). In exposed mice, a significant proportion of early born (E12) cells were found in bin 7, displaced from lower layers toward the middle layers. After injections at E16, the BrdU+ cells in control brains were found in bins 2 and 3, corresponding to layers 2 and 3 as would be expected (see Figure 2c – lower power view). Examination of the CO treated brains revealed that substantial BrdU+ cells were found in bins 4 and 5, also displaced toward the central layers of cortex.

Prenatal CO altered interneuron distribution

BrdU birthdating is an effective technique to map the disposition of neurons born on specific days. We first confirmed that the majority of BrdU+ cells were neuronal, we immunostained with the neuronal marker NeuN (Figure 3). We found that over 70% of BrdU+ cells were neurons. To determine the phenotype of displaced cells, we examined the distribution of cells likely to be projection neurons using an antibody targeting MAP2 (Curtetti *et al.*, 2002) and the distribution of interneurons using GAD-65/67. In untreated cortex, MAP2+ neurons were found in all cortical layers, with higher numbers in bins 2 and 3, which correspond to layers 2/3. A similar distribution was found in the cortex of animals treated with CO. Prenatal exposure to CO did not affect the overall distribution of cells immunoreactive for MAP2 (Figure 4.) However, the distribution of interneurons showed differences when comparing the normal versus CO treated brains. Although the overall patterns were similar, exposed

mice had a increase in interneurons in upper layers (bin 3) and relative decrease in deeper layers (bin 7,8) of the cortex (Figure 5b).

To further determine if specific subsets of interneurons were affected by CO treatment, control and treated brains were immunoreacted with antibodies directed against calretinin or parvalbumin. CR⁺ interneurons had a significant reduction in the proportion of cells in the upper layers, specifically in bin 2 (Figure 6b). PV⁺ cells had significant differences in distribution with an increase in proportion in upper layers (bin 2) and corresponding decrease in lower layers (bin 6) (Figure 7b).

Both early born and later born interneurons are affected

We assessed the distribution of cells double labeled for immunoreactivity with BrdU and MAP2, GAD 65/67, CR, and PV. Cells immunoreactive for MAP2 and BrdU had similar distributions in both treated and control mice. In contrast, double-labeled cells for GAD 65/67 and BrdU had altered distributions. E12 BrdU/GAD cells were significantly displaced towards middle layers, with an increase in proportion in bin 7 (Figure 5c). E16 BrdU/GAD cells were also significantly displaced, with an increase in proportion in bin 4 (Figure 5d). Subpopulations of interneurons were also affected. The distribution of E12 BrdU/CR⁺ cells was significantly altered in treated mice, with an increase in proportion towards middle layers (bin 8) (Figure 6c). E16 born CR⁺ cells were significantly displaced in treated mice with an increase in proportion in bin 3 (Figure 6d). E12 born PV⁺ cells were predominantly located in deeper layers in control animals, however were altered in treated mice, with an increase in bin 7

(Figure 7c). E16 born PV+ cells were proportionally reduced in bin 2 in treated mice (Figure 7d).

Prenatal exposure to CO produces changes in behavior

To determine the functional outcome of CO exposure, adult mice were tested for three behavioral paradigms: the open-field test, pre-pulse inhibition test, and hot plate test. In general, treated mice appeared remarkably similar to controls in overall locomotor behavior. They did not possess any gross motor deficits or robust behavioral differences. When assessed in the open field, treated mice behaved similar to controls in a variety of parameters including overall activity, distance traveled and time spent moving. Treated mice did not exhibit overt signs of anxiety, as they spent similar times in the middle of the field compared to controls. (Figure 8a). Mice were also tested for sensorimotor gating using the pre-pulse inhibition test. During a fifteen minute trial, each mouse received an acoustic startle stimulus of 110 dB, paired with a pre-pulse of 60, 72, or 90 dB. Control mice successfully inhibited their response consistently by approximately 20%; however treated mice did not possess significant pre-pulse inhibition (Figure 8b). In the hot plate test, control mice elicited a paw withdrawal response latency of 32.7 ± 1.8 s. Treated mice had significantly lower nociceptive thresholds, with a mean response of 22.6 ± 1.5 s (Figure 8c).

Discussion

The results from cortical wall and laminar measurements, cellular distribution measurements, and behavioral tests show that exposure to low concentrations of CO during development affects both the structure and function of the cerebral cortex. Structurally, CO affects the laminar fate of both early born and later born cells as shown in Figure 2. Furthermore, the affected cells are predominantly inhibitory interneurons and not likely projection neurons based on colocalization of BrdU and GAD 65/67 in displaced cells, shown in Figure 4. Functionally, mice exposed to CO *in utero* have long-term consequences evident by diminished sensorimotor gating and alterations in nociceptive response indicated in Figure 8. Thus low levels of CO, similar to maternal smoking, are sufficient to produce subtle structural changes in the cerebral cortex and functional deficits in offspring.

Previous studies report that prenatal exposure to CO produces a number of neurological effects. Maternal exposure to low levels of CO disrupts development of the medulla (Tolcos *et al.*, 2000), basal ganglia (Daughtrey and Norton, 1982), cerebral cortex (Ginsberg and Myers, 1974), and the cerebellar cortex (Benagiano *et al.*, 2005), as well as the development of important myelinating oligodendrocytes (Weiss *et al.*, 2004). In adult rats exposed to CO prenatally, there is a disruption in long-term potentiation in the hippocampus, suggesting long term deficiencies in learning and memory (Mereu *et al.*, 2000). At low concentrations (75-150 ppm) CO induced a variety of neurobehavioral abnormalities in rat offspring including behavioral activity levels through preweaning, delayed development of homing behavior and negative geotaxis, altered ontogeny of emotional responsiveness, and permanent cognitive deficits

(Fechter and Annau, 1977, 1980, Mactutus and Fechter, 1984, 1985, Di Giovanni *et al.*, 1993). Prenatal CO exposure also affects a variety of neurotransmitters and enzymes in the developing brain. Cagiano *et al.* demonstrated in male rats that low CO concentrations elicited changes in mesolimbic dopaminergic function and impaired sexual behavior (Cagiano *et al.*, 1998). Storm and Fechter found changes in catecholamines in the cerebellum and linked these changes with deficiencies in motor test performance and in assessments of learning and memory (Storm and Fechter, 1985). Storm *et al.* noted a decrease in total GABA content in cerebelli of 10 day old rats exposed to CO prenatally, suggesting GABAergic neurons may represent a subpopulation of neurons particularly vulnerable to CO toxicity (Storm *et al.*, 1986). Benagiano *et al.* later demonstrated layer specific decreases in GABA content in the cerebellum, as supporting evidence of motor impairments produced (Benagiano *et al.*, 2005). Our data support the toxicity of low concentrations of CO on the developing brain, and suggest a potential specificity affecting the process of neuronal migration.

Our data support previous reports of adverse effects in brain development associated with prenatal exposure to low levels of CO. The anatomical changes noted with altered laminar fate and distribution of interneurons indicated in Figures 2 and 5, suggest that interneurons are selectively vulnerable to insult during development. Selective vulnerability of this subpopulation has also been noted in other models of maternal exposure including cocaine (Crandall *et al.*, 2004), bisphenol (Nakamura *et al.*, 2007), and ethanol (Cuzon *et al.*, 2008). We also noted that of two subpopulations of interneurons: CR- or PV-expressing cells were also affected (Figures 6 & 7). CR expressing interneurons arise from

the caudal ganglionic eminence (CGE), and have a unique “outside-in” pattern of migration through the upper layers to their target layer (Rymar and Sadikot, 2007). They can exhibit either a bipolar or multipolar morphology with vertical dendritic arbors spanning the upper layers 2 & 3 of the neocortex (Jacobowitz and Winsky, 1991). Selective loss of CR⁺ interneurons is linked to epilepsy and decreased inhibition in the cortex (Cobos *et al.*, 2005), PV expressing interneurons arise from the MGE are a unique subpopulation of interneurons based on morphology and activity. They reside predominantly in middle to deeper layers of the cortex, have unique basket-like morphology, and are generally fast-spiking in firing activity (Kawaguchi and Kondo, 2002). In contrast to PV⁺ interneurons, CR⁺ cells are not fast spiking, rather are heterogeneous in their firing patterns (Kawaguchi and Kubota, 1997). Mice exposed to CO *in utero* had altered distributions of both PV⁺ and CR⁺ neurons. The data in Figure 7 show a significant reduction in the proportion of PV⁺ interneurons in deeper layers and coincident increase in upper layers. CR⁺ cells were proportionally reduced in upper layers of the cortex indicated in Figure 6. This suggests that while CO can selectively affect interneurons, the effects are noted in neurons arising from either the MGE or the CGE. Furthermore, these displaced cells may have functional effects altering the overall excitatory/inhibitory tone throughout the cortex.

Prenatal CO exposure also results in functional changes in offspring. We did not note differences in overall locomotor activity in treated mice compared to controls. This was not surprising, as the differences noted in interneuron distribution were subtle, and not detected with a gross behavioral test. We chose to examine the mice with more sensitive measures, specifically the pre-pulse

inhibition test and hot plate test. The pre-pulse inhibition test is a measurement of sensorimotor gating that engages many brain structures, including somatosensory, insular, and cingulate cortices (Neuner *et al.*, 2010). This test is highly conserved across mammals, and considered one of the most translatable tests from mouse to human (Crawley *et al.*, 1997). Mice exposed to CO *in utero* did not have pre-pulse inhibition shown in Figure 8b. This phenomenon is also seen in schizophrenic patients, who also have altered interneuron distribution (Raghanti *et al.*, 2010) and lack pre-pulse inhibition (Braff *et al.*, 1978, Kumari *et al.*, 2000). The hot plate test was used to assess supraspinal mediated pain processing that involves somatosensory cortex. Previous studies have shown a critical role of GABAergic transmission in supraspinal mediated pain processing. In a mouse knockout model, researchers found that mice lacking GAD-65 demonstrated hyperalgesia (Kubo *et al.*, 2009). In another transgenic mouse model, mice were bred to overexpress the GABA transporter GAT-1, thereby altering the tonic GABA levels in the synaptic environment. These mice exhibited hyperalgesia to a variety of thermal and chemical nociceptive challenges (Hu *et al.*, 2003). In our study, mice exposed to CO *in utero* had significantly shorter hot plate latencies compared to controls (Figure 8c), consistent with previous reports that alterations in GABAergic activity can influence nociception.

The mechanism mediating this effect remains to be studied. The major effect of CO is attributed to its ability to produce hypoxia by binding to hemoglobin forming carboxyhemoglobin and preventing O₂ delivery to tissues. CO readily crosses the placenta and binds with fetal hemoglobin with a higher affinity than adult hemoglobin (Longo, 1970). During chronic exposure, the gas may accumulate in the tissues and act as a signaling molecule. Many

hypotheses on CO signaling arise from the evidence that CO participates in many of the NO-mediated signaling pathways. One such pathway is the NO-mediated activation of soluble guanylate cyclase, the enzyme that catalyzes the formation of cyclic guanosine mono-phosphate (cGMP). cGMP is an important small molecule for neuronal migration. In insect models of neuronal migration, Haase and Bicker showed that pharmacological inhibition of endogenous nitric oxide synthase (NOS), soluble guanylate cyclase (sGC), and cGMP-dependent kinase (cGK) each significantly decreased neuronal migration (Haase and Bicker, 2003). The developmental expression profile of soluble guanylate cyclase in the mouse also parallels the period of neuronal migration in the developing brain (Ding *et al.*, 2005). Furthermore, mice with deletion of the gene for sGC have abnormal neocortical development consistent with deficiencies in neuronal migration (Demyanenko *et al.*, 2005). Another study used a model of fetal alcohol syndrome in mice and found that the inhibition of neuronal migration by ethanol was reversed by either increasing cGMP levels or decreasing cAMP levels (Kumada *et al.*, 2006). In the mouse embryo, tangentially migrating neurons from the GE naturally produce cGMP earlier than neurons situated in the cortex, and also produce cGMP in response to exogenous NO treatment (Currie *et al.*, 2006). The evidence that cGMP is critical for neuronal migration suggests that alterations of cGMP levels may dramatically affect neuronal migration and neocortical development. Our laboratory has recently studied this effect and determined that gestational exposure to CO results in a decrease in cGMP levels in pups at birth (unpublished data).

In conclusion, we report that prenatal CO exposure has neurological consequences in offspring that parallels some components of neurological

diseases. Maternal smoking associates with a number of neurological consequences such as autism and attention deficit disorder (Agrawal *et al.*, Milberger *et al.*, 1996, Milberger *et al.*, 1998, Larsson *et al.*, 2005). We have now shown a relationship with prenatal CO and cortical dysplasias, which are connected to schizophrenia and epilepsy (Reiss-Zimmermann *et al.*, Reynolds and Beasley, 2001). Autistic patients have altered numbers of parvalbumin expressing interneurons (Casanova *et al.*, 2003). Variation in the overall excitatory/inhibitory tone of the cerebral cortex caused by altered interneuron distribution underlies many forms of epilepsy (Badawy *et al.*, 2007). The results of our study show parallel changes in both structure and function of the cerebral cortex after prenatal CO exposure with some aspects of these neurological diseases. Our data show that exposure to low concentrations of CO similar to what is noted in heavy smokers results in altered laminar fate of cells. The majority of displaced cells are interneurons. The overall distribution of interneurons throughout the cortex is also altered in exposed mice compared to controls. Mice exposed to CO *in utero* also lack pre-pulse inhibition to the acoustic startle reflex and are significantly hyperalgesic. This study demonstrates another toxic effect of maternal smoking that can produce permanent structural and functional consequences in the developing fetus.

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Figure 1. Cresyl violet stained section of somatosensory cortex.

Pregnant dams were exposed to either 0 or 150 ppm CO from E7 until birth. For cortical measurements, pups were examined at P4, P11, or as adults (P56 or later), 40 μ m thick coronal sections containing somatosensory cortex were stained with cresyl violet. The cortical thickness and layer thicknesses were measured in a region approximately 2 mm lateral to the midline.

Figure 1. Cresyl violet stained section of somatosensory cortex.

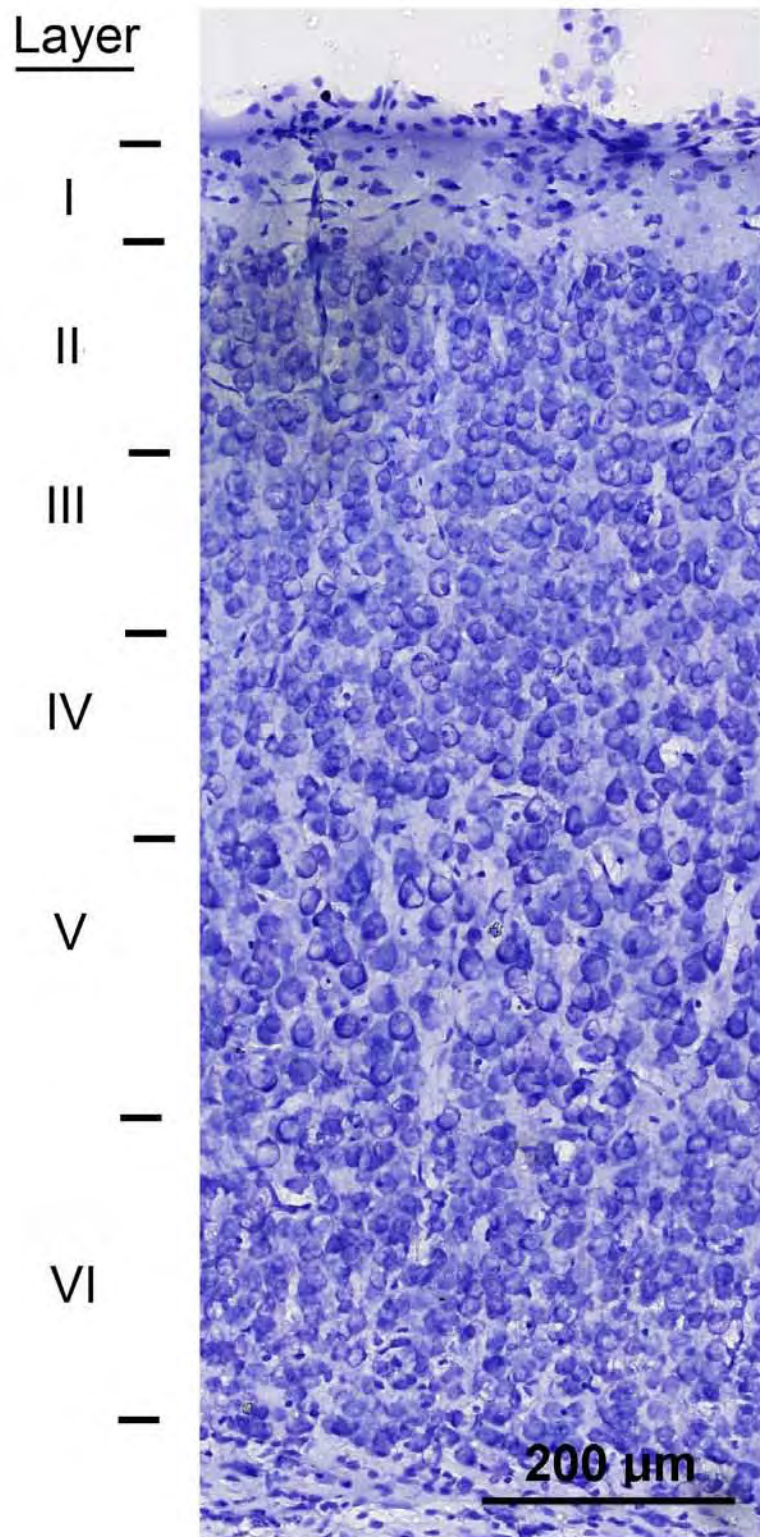


Table 1. The effects of prenatal CO on cortical wall and laminar thickness.

Mice were exposed to 0 or 150 ppm *in utero* and examined at P4, P11, or as adults (P56 or later). The overall cortical thickness and laminar thicknesses were measured on cresyl violet stained sections of somatosensory cortex. At P4, CO treated mice had significantly thinner cerebral walls. The relative layer thicknesses were proportional to the overall thickness of the cortex. By P11, CO treated mice recovered, and had similar cortical thicknesses compared to controls. This effect was true as adults, with no significant gross anatomical differences between treated and controls. Layers of neocortex are indicated with Roman numerals.

Table 1. The effects of prenatal CO on cortical wall and laminar thickness.

Age	<u>0 ppm</u>				<u>150 ppm</u>			
	<u>cortical thickness</u>	<u>layer</u>	<u>layer thickness (μm)</u>	<u>layer proportion (%)</u>	<u>cortical thickness</u>	<u>layer</u>	<u>layer thickness (μm)</u>	<u>layer proportion (%)</u>
P4	772 ± 20.1	I	47.1 ± 4.7	6.1 ± 0.7	* 707.5 ± 38.7	I	38.6 ± 3.2	5.6 ± 0.3
		II-IV	200.7 ± 17.3	26.9 ± 3.2		II-IV	178.2 ± 11.5	24.9 ± 0.5
		V	220.5 ± 3.2	28.5 ± 1.5		V	232.2 ± 18.1	31.8 ± 0.3
	782.6 ± 40.3	VI	277.7 ± 1.5	36.6 ± 1.8	773.2 ± 17.8	VI	248.5 ± 19.4	37.2 ± 1
		I	57 ± 2.9	7.9 ± 0.5		I	56.3 ± 1.3	7.5 ± 0.1
		II-IV	263.7 ± 13.6	36.3 ± 2.1		II-IV	260 ± 6	34.6 ± 0.7
P11		V	225.6 ± 11.7	31.2 ± 1.8		V	223.8 ± 5.1	29.7 ± 0.6
		VI	235.3 ± 12.1	32.4 ± 1.9		VI	232.5 ± 5.3	30.8 ± 0.6
	779.6 ± 21.1	I	89.4 ± 3.1	12.2 ± 0.3	787.2 ± 12.4	I	94.2 ± 1.9	12 ± 0.4
		II-IV	294.1 ± 10.2	40.1 ± 1		II-IV	309.9 ± 6.2	39.6 ± 1.2
		V	197.7 ± 6.8	27 ± 0.6		V	208.4 ± 4.1	26.6 ± 0.8
		VI	181.7 ± 6.3	24.8 ± 0.6		VI	191.4 ± 3.8	24.5 ± 0.8

* $p < 0.05$, ANOVA, followed by Holm-Sidak post hoc test

Figure 2. Prenatal CO exposure results in displaced cells born on either E12 or E16.

(a) illustrates higher power views of BrdU immunoreactivity in the cortex of adult mice injected with BrdU *in utero* on E12 or E16. (b) and (c) show the distributions of BrdU+ cells in the cortex of animals injected on E12 or E16. (d) and (e) show the distributions of BrdU+ cells through the thickness of the cortex. To obtain these values, a 500 μm wide section of somatosensory cortex was divided into 10 equal bins, with bin 1 corresponding to the area closest to the pia and bin 10 closest to the white matter. BrdU+ cells were counted in each bin and plotted as a percent of the total number of BrdU+ cells. Prenatal CO administration results in a significant proportion of displaced cells born early (E12) and later (E16) in corticogenesis. In both cases, displaced cells were located towards middle layers of the cortex. (n=4 mice, 3 slices/mouse; * $p < 0.05$; Two-way ANOVA, followed by Holm-Sidak post hoc test)

Figure 2. Prenatal CO exposure results in displaced cells born on either E12 or E16.

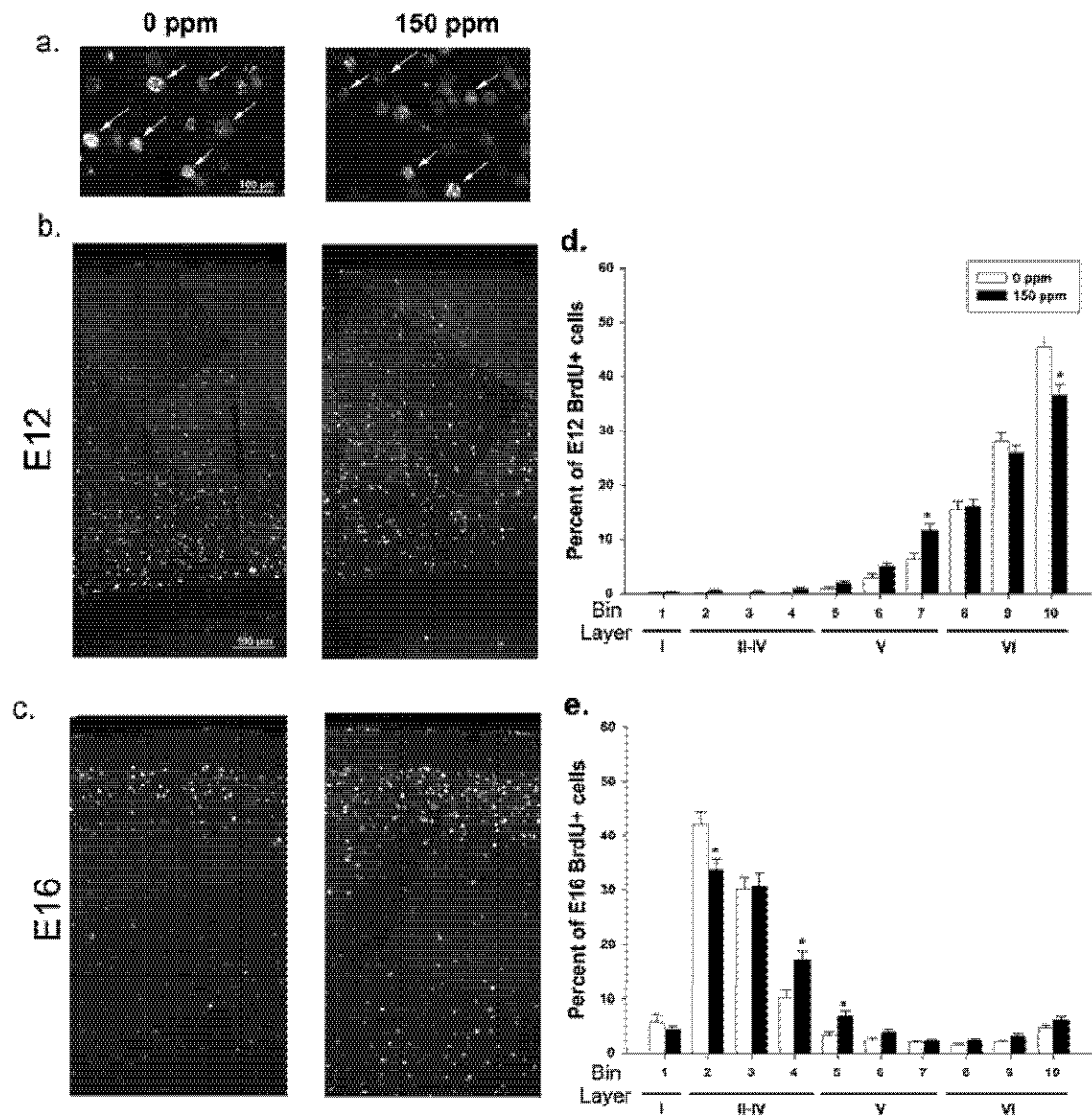


Figure 3. The majority of BrdU labeled cells colocalize with NeuN

(a) is an example of cells immunostained for the neuronal marker NeuN (red) and BrdU (green). (b) shows the disposition of cells immunostained for both NeuN and BrdU. Over 70% of BrdU+ cells were co-labeled with NeuN.

Figure 3. The majority of BrdU labeled cells colocalize with NeuN

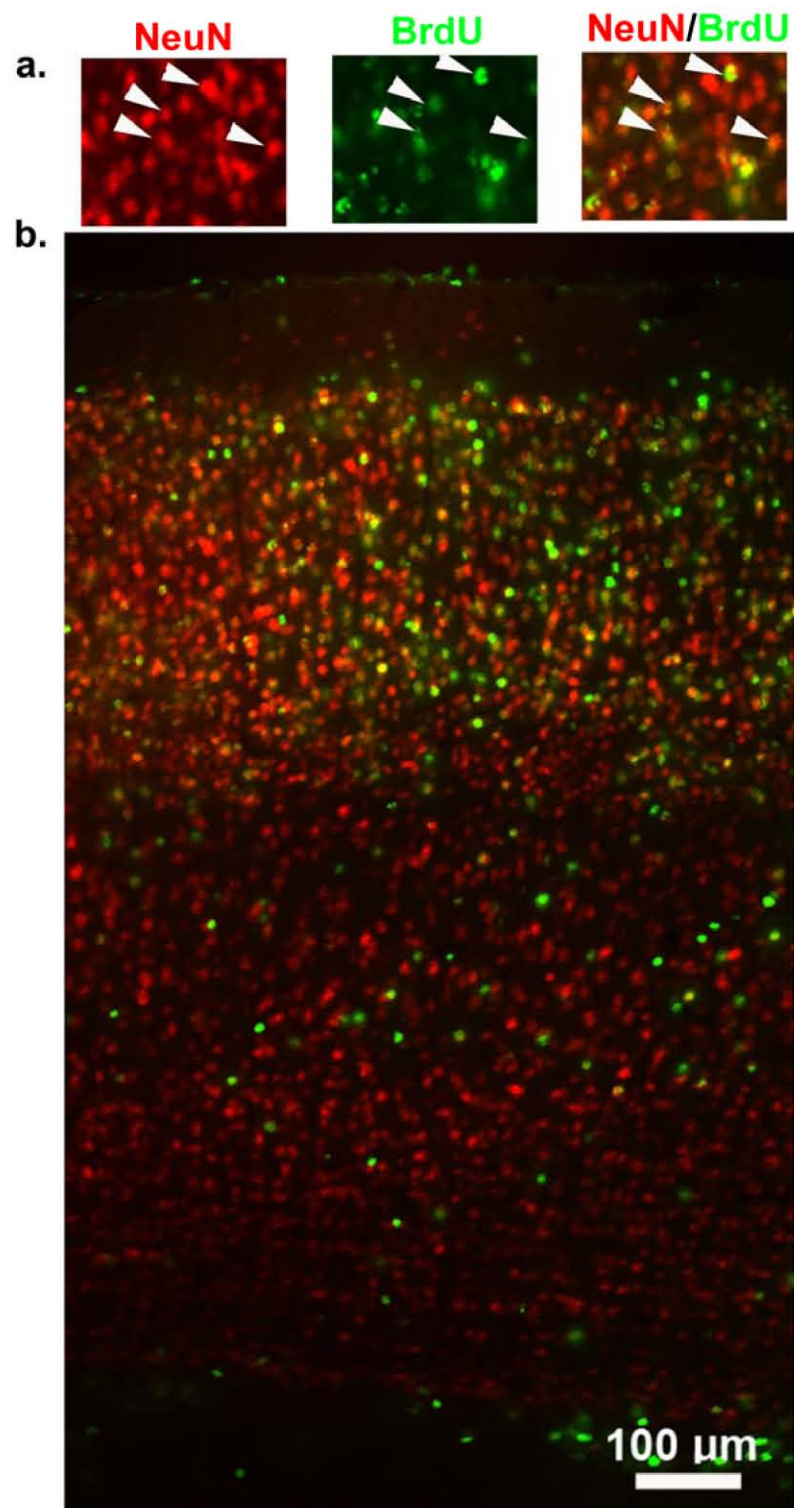


Figure 4. Prenatal CO exposure does not affect the distribution of projection neurons.

(a) is an example of cells in the somatosensory cortex of mice immunoreactive for BrdU and MAP2. Arrows point to the same cells that are positive for MAP2, BrdU or both markers. (b) shows the distribution of MAP2+ cells through the thickness of the somatosensory cortex in control and CO treated cortex. Overall, the distribution of projection neurons was not significantly different in treated mice compared to controls. (c, d) Treated mice also had similar distributions of both early born (E12) and later born (E16) projection neurons. (n=4 mice, 3 slices/mouse; * $p < 0.05$; Two-way ANOVA, followed by Holm-Sidak post hoc test).

Figure 4. Prenatal CO exposure does not affect the distribution of MAP2 positive cells.

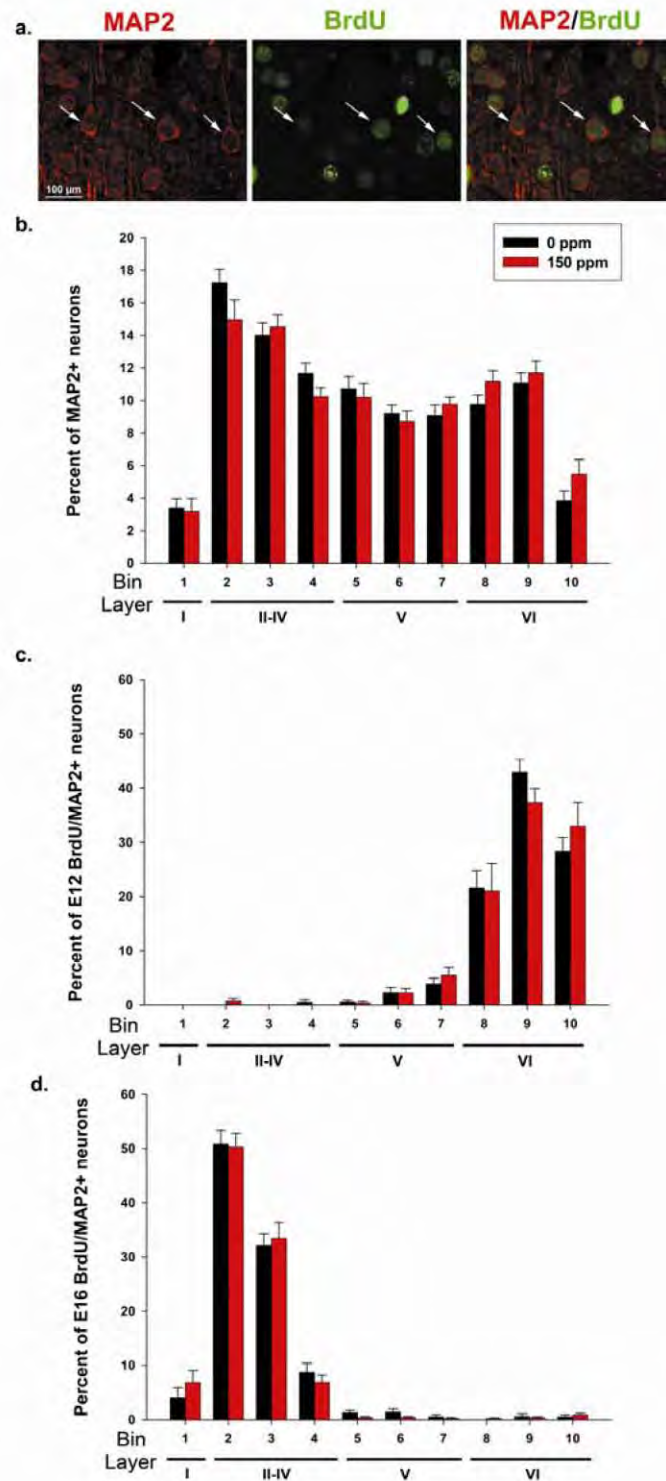


Figure 5. Prenatal CO exposure affects the distribution of interneurons.

(a) is an example of cells in the somatosensory cortex of mice immunoreactive for BrdU and GAD 65/67. Arrows point to the same cells that are positive for GAD, BrdU or both markers. (b) shows the distribution of GAD 65/67+ cells through the thickness of the somatosensory cortex in control and CO treated cortex. Overall, the distribution of interneurons was significantly different in treated mice compared to controls. Treated mice had a significant proportion of interneurons in upper layers and layer 6, with respective decrease proportion in middle layers. (c, d) Treated mice also had altered distributions of both early born (E12) and later born (E16) interneurons. (n=4 mice, 3 slices/mouse; * $p < 0.05$; Two-way ANOVA, followed by Holm-Sidak post hoc test).

Figure 5. Prenatal CO exposure affects the distribution of interneurons.

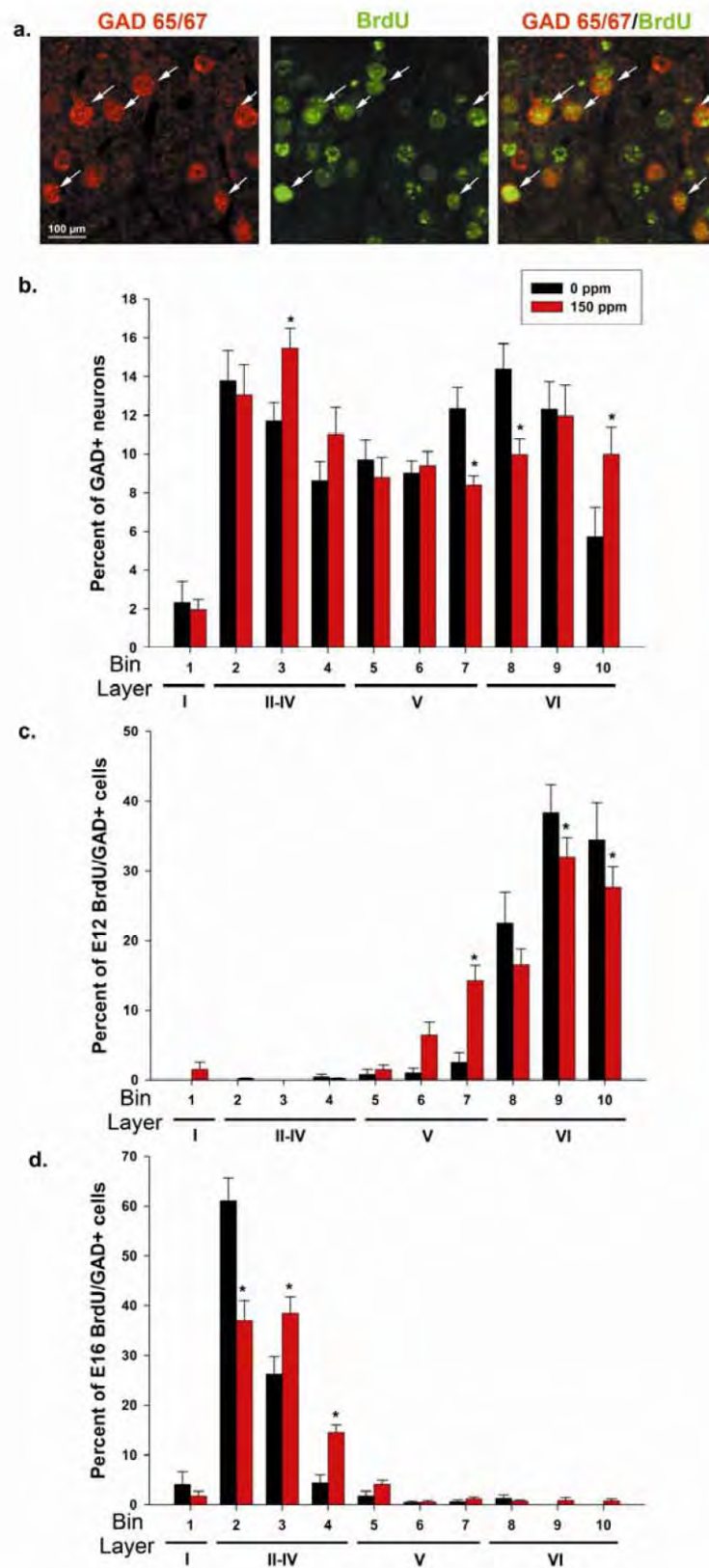


Figure 6. Prenatal CO exposure affects the distribution of CR⁺ interneurons.

(a) is an example of cells in the somatosensory cortex of mice immunoreactive for BrdU and CR. Arrows point to the same cells that are positive for CR, BrdU or both markers. (b) shows the distribution of CR⁺ cells through the thickness of the somatosensory cortex in control and CO treated cortex. Overall, the distribution of CR⁺ interneurons was significantly different in treated mice compared to controls. Treated mice had a significant decrease in proportion of CR⁺ cells in bin 3 (c, d) Treated mice also had altered distributions of both early born (E12) and later born (E16) CR⁺ interneurons displaced towards middle layers. (n=4 mice, 3 slices/mouse; * $p < 0.05$; Two-way ANOVA, followed by Holm-Sidak post hoc test).

Figure 6. Prenatal CO exposure affects the distribution of CR⁺ interneurons.

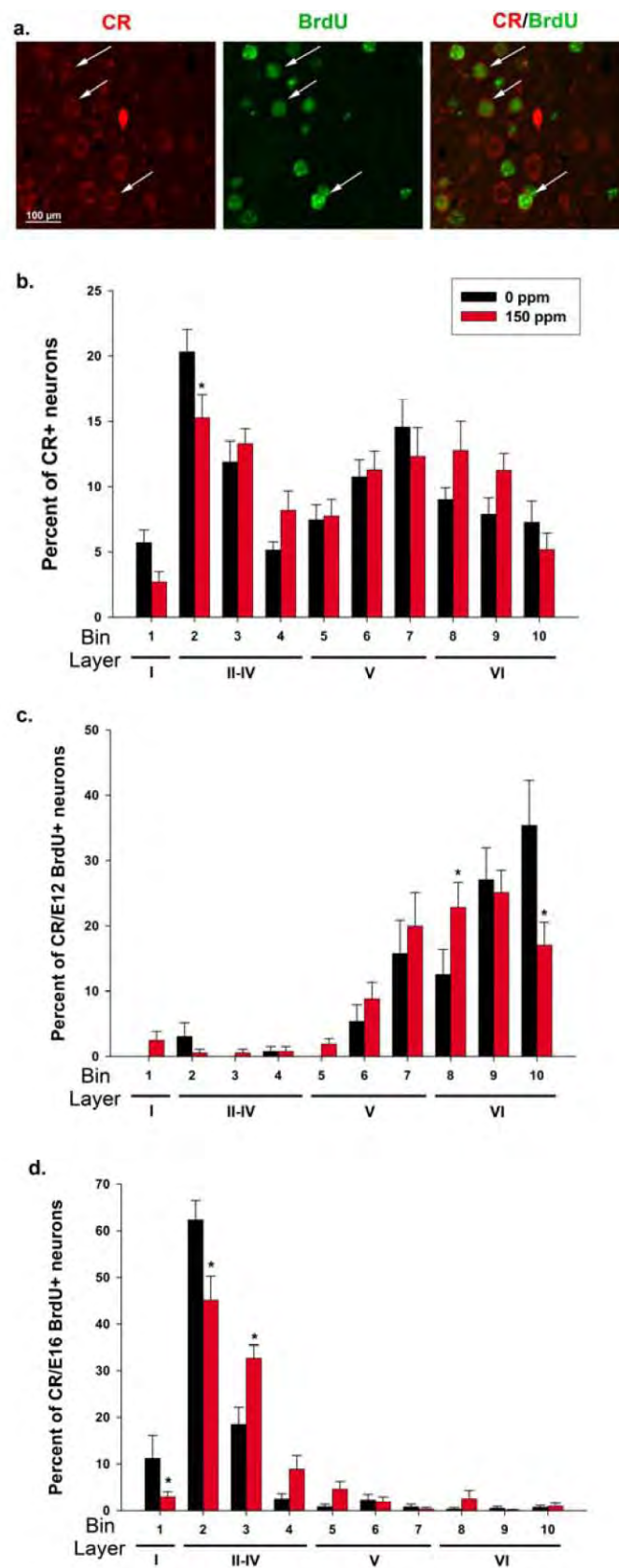


Figure 7. Prenatal CO exposure affects the distribution of PV⁺ interneurons.

(a) demonstrates an example of cells in the somatosensory cortex of mice immunoreactive for BrdU and PV. Arrows point to the same cells that are positive for PV, BrdU or both markers. (b) shows the distribution of PV⁺ cells through the thickness of the somatosensory cortex in control and CO treated cortex. Control mice have the majority of PV⁺ cells distributed in bins 6 & 7. CO treated mice have altered distributions with an increase in bin 2 and corresponding decrease in bin 6. (c, d) Treated mice also had altered distributions of both early born (E12) and later born (E16) PV⁺ interneurons displaced towards middle layers. (n=4 mice, 3 slices/mouse; * $p < 0.05$; Two-way ANOVA, followed by Holm-Sidak post hoc test).

Figure 7. Prenatal CO exposure affects the distribution of PV⁺ interneurons.

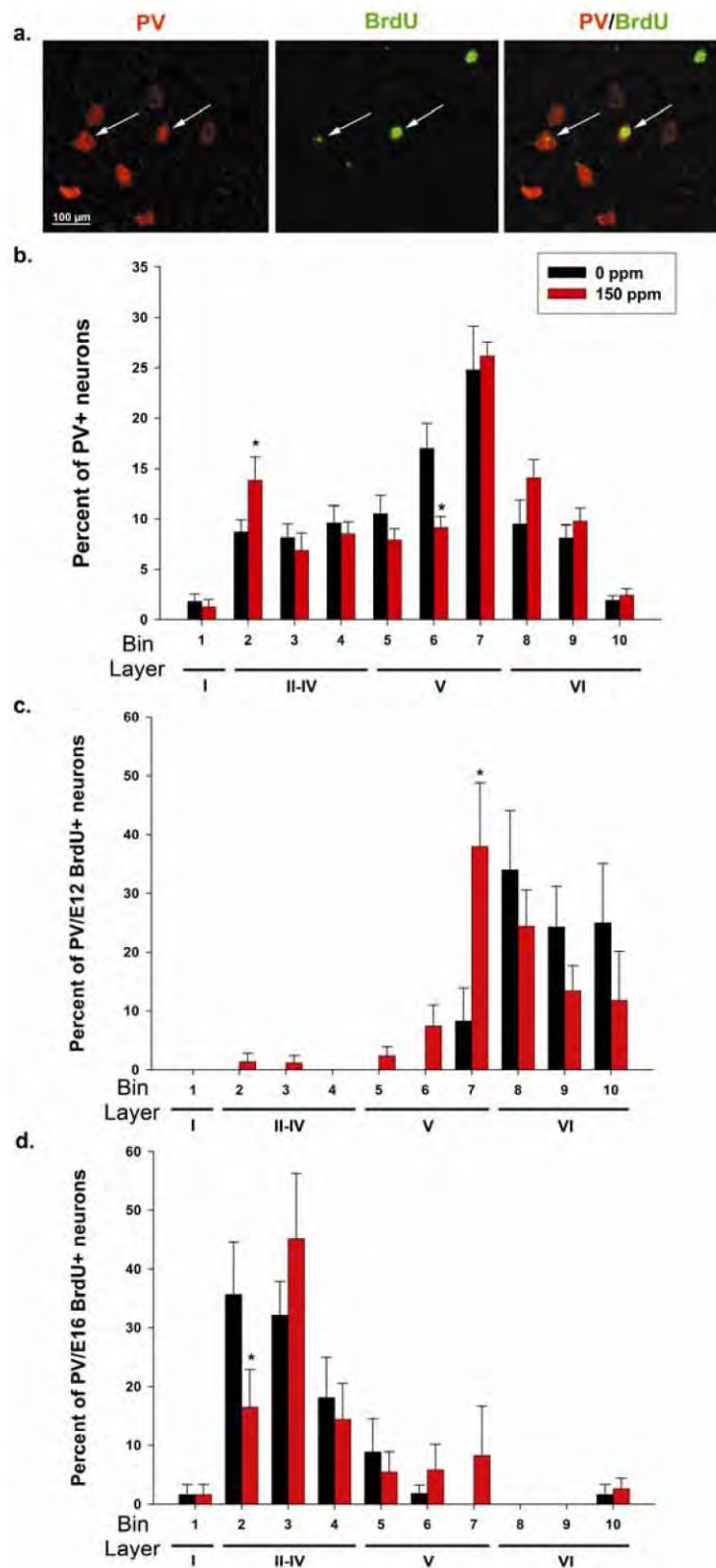
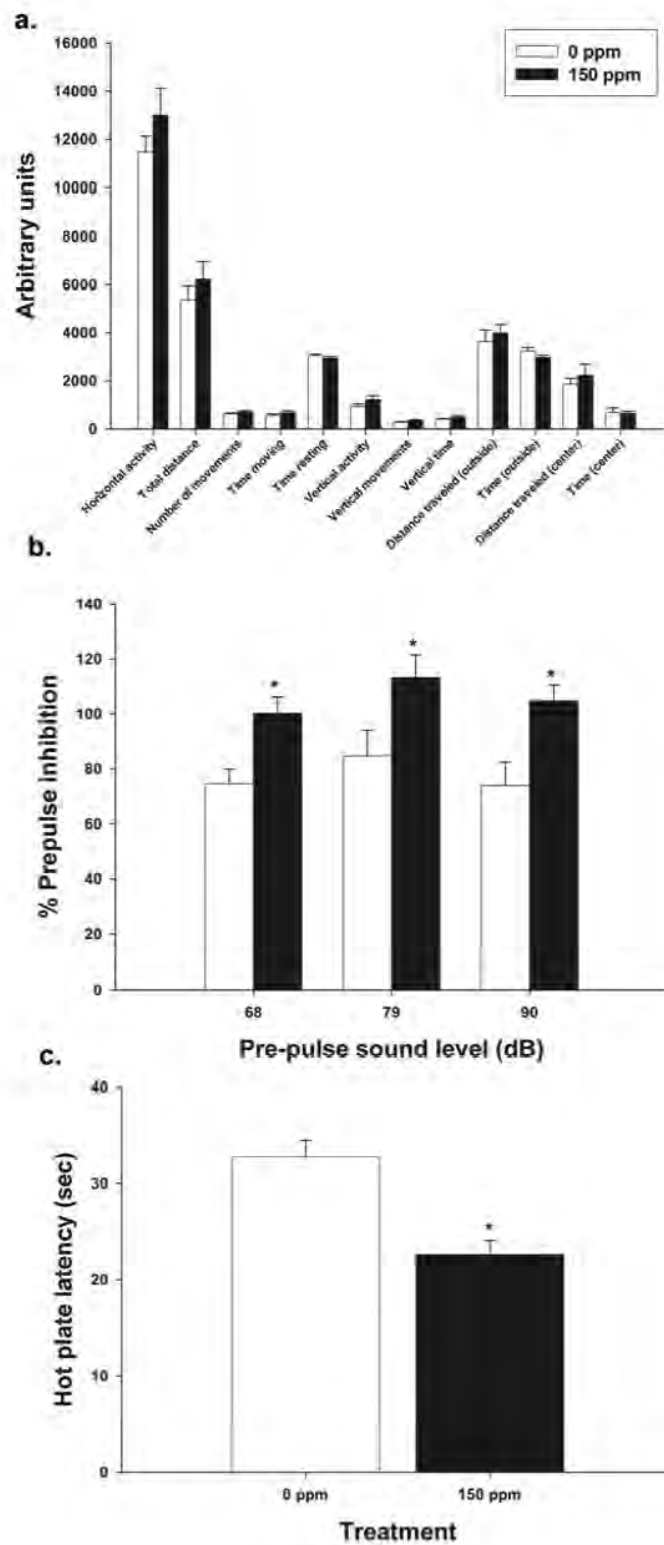


Figure 8. Prenatal CO results in functional deficits in adult mice exposed *in utero*.

Adult mice exposed to either 0 or 150 ppm *in utero* were subjected to a variety of behavioral tests. (a) Treated mice performed similar to controls in measurements of overall locomotion and activity. (b) In the pre-pulse inhibition test, control mice demonstrated approximately 80% inhibition to the acoustic startle stimulus (110 dB) when presented with pre-pulse stimuli (68, 79, or 90 dB). Treated mice failed to demonstrate significant pre-pulse inhibition with any of these pre-pulse stimuli ($n=7$, $p < 0.05$, Student's t-test). (c) In the hot plate test, control mice exhibited nocifensive behavior (scratching or licking the hindpaw) after 32.6 ± 1.7 sec. Treated mice showed hyperalgesia, exhibiting nocifensive behavior significantly sooner than controls (22.6 ± 1.5 sec; $n=7$, $p < 0.05$, Student's t-test).

Figure 8. Prenatal CO results in functional deficits in adult mice exposed *in utero*.



CHAPTER 4

Carbon monoxide directly impairs interneuron migration into the neocortex

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Abstract

Prenatal exposure to carbon monoxide, the toxic gas formed by incomplete combustion of organic material, affects the development of several brain structures including the brainstem, cerebellum and cerebral cortex. While the effects of acute CO toxicity have been well studied, the effect of chronic low level CO on neuronal migration and development of the cerebral cortex remains a significant gap in the literature. Furthermore, the direct effects of CO as a signaling molecule in migrating interneurons have not been studied. In this study, we exposed pregnant dams to 0 or 150 ppm CO from E7 until birth. Chronic exposure to CO resulted in a decrease of second messenger cGMP and a decrease in a downstream target, phosphorylated VASP in brains of mouse pups exposed *in utero*. In subsequent experiments, we labeled cells in the source of interneurons (the ganglionic eminence) of E15 organotypic slices with Dil and incubated for 3 days in normal and 50 ppm CO environments. The distribution, orientation, and length of leading processes of migrating interneurons were measured. In CO treated slices, fewer migrating interneurons reached the cortical plate as compared to controls (53.9 ± 7.1 vs. $13.8 \pm 3.5\%$). Furthermore, the length of leading processes of cells was significantly shorter in treated slices compared to controls (64.3 ± 1 vs. $78.4 \pm 0.7 \mu\text{m}$). When grown in the presence of the phosphodiesterase inhibitor IBMX, a greater proportion of cells reached the cortical plate ($32.3 \pm 3.5\%$) and the diminished leading process length was recovered ($82.2 \pm 1.1 \mu\text{m}$). These data indicate that CO acts as a signaling molecule and impairs neuronal migration by acting through the CO/NO-cGMP pathway.

Keywords:

Carbon monoxide, neuronal migration, interneuron, cGMP, IBMX

Introduction

Development of the cerebral cortex is a precisely regulated and complex process involving the proliferation, migration and differentiation of neurons. The six layers of the cortex develop in successive waves of cellular migration in two main patterns. The timing of these migration patterns is well characterized through cell lineage and cell fate experiments in a variety of mammals, including humans, ferrets and rodents (Hatten, 1999, Marin and Rubenstein, 2003, Kriegstein, 2005, Ayala *et al.*, 2007). Radial migration occurs when projection neurons migrate along radial glia that extend from the ventricular zone following an orthogonal orientation from this region. In contrast, interneurons migrate tangentially from the ganglionic eminences of the ventral telencephalon roughly perpendicular to the direction of radial migration (Marin and Rubenstein, 2003). Inhibitory interneurons migrating tangentially rely on a number of intrinsic and extrinsic signals that stimulate motility and guide the cell to its location. Their complex migration pattern requires a succinct coordination of cytoskeletal reorganization in response to extrinsic signaling cues. As a result, these neurons are particularly vulnerable to insult, and perturbation of migration has profound effects on the structure and function of the cerebral cortex.

Neuronal migration disorders occur when either genetic or environmental factors affect this process and result in cortical dysplasia (Taylor *et al.*, 1971, Tassi *et al.*, 2002). Exposure to environmental toxins such as cigarette smoke, alcohol, methyl mercury, or radiation can result in a myriad of cortical defects based on abnormal proliferation and migration, aberrant cell death patterns, or abnormal gliogenesis (Kriegstein, 1996, Krauss *et al.*, 2003, Pang *et al.*, 2008). Carbon monoxide (CO), the toxic gas formed by incomplete combustion of

carbon rich material, is an environmental toxin affecting the development of a variety of brain structures including the medulla, basal ganglia, cerebellum, and cerebral cortex (Ginsberg and Myers, 1974, Daughtrey and Norton, 1982, Tolcos *et al.*, 2000, Benagiano *et al.*, 2005). The most common sources of CO exposure are cigarette smoke, vehicle exhaust, and poorly ventilated heating sources or stoves. CO primarily exerts its toxic effect by binding to hemoglobin producing hypoxia. CO can also act as a gaseous signaling molecule similar to nitric oxide (NO). At elevated intracellular levels, CO can bind to important heme containing molecules in the cell including mitochondrial cytochromes of the electron transport chain, cytochrome P450, enzymes of metabolism, myoglobin, neuroglobin and cytoglobin (Caughey, 1970, Sawai *et al.*, 2005, Fago *et al.*, 2006). The signaling role for CO first emerged from observations in smooth muscle cells that CO can stimulate soluble guanylate cyclase to produce cGMP (Furchgott and Jothianandan, 1991). cGMP is an important second messenger for a number of developmental processes, including formation of the neuromuscular junction (Wang *et al.*, 1995), modulation of gap junctions in the neocortex (Rorig and Sutor, 1996), neural plate responsiveness to Sonic hedgehog signaling (Robertson *et al.*, 2001), and neuronal growth cone extension and responsiveness to semaphorin 3A (Song *et al.*, 1998, Van Wagenen and Rehder, 2001). cGMP also activates cGMP dependent kinases (cGK's), which phosphorylate important cytoskeletal proteins involved in neuronal migration, such as the actin associated cytoskeletal complex Ena/VASP (Goh *et al.*, 2002). These processes are essential for brain development under normal conditions.

While many of the effects of CO have been studied in relationship to brain development, few have studied the effects on the migration of cells into the developing neocortex. In this study, we used both *in vivo* exposures to CO to detect global changes in the brains of pups and *in vitro* organotypic slices to study the direct effects of CO on the migration of interneurons into the developing neocortex. We report that gestational exposure to CO results in a decrease of cGMP as well as a decrease in its downstream target, phosphorylated VASP. Furthermore, *in vitro* exposure to CO results in impaired migration and shorter leading processes of migrating cells. This effect is rescued by the phosphodiesterase inhibitor IBMX, suggesting the CO/NO-cGMP-cGK pathway participates in this effect.

Materials and Methods

Animals and Exposures

All animal work was approved by the Uniformed Services University Institutional Animal Care and Use Committee and in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 86-23, revised 1987). Timed pregnant mice (CD-1) were purchased from Charles River Laboratories (Wilmington, MA). For gestational exposures, pregnant dams were placed in sealed acrylic environmental chambers from E7 until birth. A CO concentration of 150 ppm was maintained by mixing 7.5% CO with room air with the flow controlled by meters and monitored with a calibrated meter (Interscan RM14-500M or 50.0 M, Chatsworth, CA). For *in vitro* experiments, slices were maintained in an incubator (95% O₂, 5% CO₂, 37°C) at either 0 or 50 ppm CO. CO levels were continuously monitored with a GasBadge Pro CO detector (Industrial Scientific, Frederick, MD) and waste gas vented into an exhaust hood.

cGMP and VASP measurements

Brains from pups exposed to 0 or 150 ppm CO from E7 to P0 were rapidly removed at birth, flash frozen and stored at -80°C. cGMP was extracted and measured using a commercially available ELISA kit according to the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). Proteins were extracted from whole brain as previously described (Jacobowitz and Heydorn, 1984) and equivalent concentrations of protein measured by the bicinchoninic acid (BCA) test were subjected to Western blot. Blots were probed for VASP and phospho-VASP using the appropriate primary antibodies (mouse anti-VASP,

1:1000; rabbit anti-phospho-VASP, 1:1000, Enzo Life Sciences, Plymouth Meeting, PA), subsequent secondary anti-bodies (anti-mouse, 1:5000; anti-rabbit, 1:5000, Li-Cor Biosciences, Lincoln, NE) and visualized using an Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

Organotypic slice cultures

Embryos were removed from normal CD-1 pregnant mice at E15 via cesarean section. Brains of embryos were removed under sterile conditions in a laminar airflow hood and cut into 350- μ m thick coronal slices containing the ganglionic eminence using a tissue chopper (Stoeling, Wood Dale, IL). During dissection, brains and slices were perfused with cold and oxygenated artificial cerebral spinal fluid (containing in mM: CaCl_2 2.4, KCl 3.2, MgSO_4 1.2, NaCl 124, NaHCO_3 26, NaH_2PO_4 1.2, glucose 10). Slices containing the ganglionic eminence (GE) were cultured on inserts (Millipore, Bedford, MA) and placed into 6-well plates in Neurobasal media (Gibco, Carlsbad, CA) containing B27, N2 and G.1.2 supplements. In some experiments, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 50 μ M Sigma-Aldrich, St. Louis, MO) was added to the media. To visualize neurons migrating tangentially, small crystals of Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, Eugene, OR) were placed in the GE. The slices incubated for 3 days in an incubator (95% O_2 , 5% CO_2 , 37°C) with 0 or 50 ppm CO , and then fixed overnight in a solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Slices were incubated with the nuclear marker bisbenzamide (0.001%) for 10 min prior to imaging on a fluorescence microscope (Zeiss, Thornwood, NY).

Neuronal migration analyses

Migrating cells from the GE were assessed on a number of parameters using Adobe Photoshop (Adobe Systems, Inc.) (Figure 1). First, borders were drawn to delineate the cortical plate from the intermediate zone. The lateral border was drawn at the corticostriate junction. The distribution of cells was counted in both the cortical plate and intermediate zone. To determine the proportion of cells orientated tangentially vs. radially, the angle of a line drawn from the cell body to the tip of the leading process was measured relative to the pial border using ImageJ software. Cells oriented perpendicular to the pia ($0 \pm 15^\circ$ or $180 \pm 15^\circ$) were considered radial. All other cells were considered tangential. For cells with multiple branches, the average angle of the branches was used. To measure the length of the leading process, a line was drawn from the cell body to the tip of the leading process and measured using ImageJ software (Girish and Vijayalakshmi, 2004). For cells with multiple branches, the length of each branch was averaged (Poluch *et al.*, 2008).

Statistical analyses

cGMP levels were compared between treated animals and controls using a Student's t-test. The ratio of phosphorylated VASP to VASP was calculated using ImageJ software and data compared using a Student's t-test. Organotypic slice data including the distribution, orientation and length of leading processes were analyzed using a Two-Way ANOVA followed by the Holm-Sidak post-hoc test for multiple pairwise comparisons. The Holm-Sidak test was chosen because

it is more powerful than the frequently used Bonferonni test for multiple paired comparisons. A p -value less than 0.05 was considered statistically significant.

Results

Prenatal exposure to CO resulted in decreased levels of cGMP and phospho-VASP

To determine if chronic low level CO exposure affected production of the secondary messenger cGMP, brains of pups from various litters exposed to CO *in utero* were assayed for cGMP using an ELISA. Mice were exposed to either 0 or 150 ppm CO from E7 until birth. cGMP was extracted from whole brain using trichloroacetic acid and assayed using an ELISA. Exposed mice had significantly lower levels of cGMP (Figure 2a: 0.85 ± 0.15 vs. 0.401 ± 0.1 pg/ml, $n=5$, $p = 0.039$). To determine if downstream targets of cGMP were affected, brains from alternate pups were assayed for proteins using Western blots. We probed for both the phosphorylated and non-phosphorylated form of VASP in the same blot, and imaged the bands simultaneously on different infrared emission channels (Figure 2b). From those images, we quantified the ratio of phosphorylated VASP to non-phosphorylated VASP. Mice exposed to CO *in utero* also had significantly lower phospho-VASP/VASP ratios (Figure 2c: 1.89 ± 0.11 vs. 1.08 ± 0.31 , $n = 5$, $p = 0.039$).

CO exposure impaired interneuron migration and resulted in shorter leading processes

To study the direct effects of CO gas on interneuron migration, E15 organotypic slices were grown in either CO (50 ppm) or control environments. Cells in the ganglionic eminence were labeled with the lipophilic fluorescent dye Dil (Figure 3). After 3 days *in vitro*, the slices were removed from the incubators

and fixed prior to imaging. In the control slices, $53.9 \pm 7.1\%$ of the labeled cells reached the cortical plate (Figure 4). In contrast, CO treated slices had a significant reduction in cells reaching the cortical plate ($13.8 \pm 3.5\%$; $p < 0.001$).

To determine if CO affected the morphology of migrating cells, the length of the leading processes of migrating cells was measured. In control conditions, the mean length of a leading process was $78.4 \pm 0.7 \mu\text{m}$ (Figure 4). Treated cells had significantly shorter leading processes ($64.3 \pm 1.3 \mu\text{m}$; $p < 0.001$).

To determine if CO altered the response of a migrating cell to the environment, we measured the angle of the leading process relative to the pia. Cells oriented $0^\circ \pm 15^\circ$ or $180^\circ \pm 15^\circ$ relative to the pia were considered radial, and all others considered tangential. In all treatment conditions, over 90% of cells were oriented tangentially (Figure 7). This was valid throughout the cortical plate and intermediate zone.

IBMX rescued CO impairment of migration, restored the leading processes of migrating interneurons, and increased branching

Since CO impaired migration of cells and the treated cells had shorter leading processes, we sought to determine if the CO/NO-cGMP-cGK pathway was affected. CO acts as a partial agonist to sGC and chronic exposure resulted in the decrease of cGMP, we added the phosphodiesterase inhibitor IBMX to the media to salvage cGMP produced endogenously. In control slices, treatment with IBMX resulted in a slight decrease in migration of cells into the cortical plate, however this was not statistically significant. In CO treated slices, IBMX treatment improved migration and resulted in a significant increase in the percent of CO treated cells reaching the cortical plate ($32.3 \pm 3.5\%$; $p=0.01$; Figure 4). To

determine whether this effect was mediated by cytoskeletal elements, we measured the length of leading processes in cells treated with IBMX and grown in the presence of CO. In CO treated slices, IBMX successfully restored the length of leading processes of migrating interneurons from $64.3 \pm 1.3 \mu\text{m}$ to $82.2 \pm 1.1 \mu\text{m}$ (Figure 5). This effect was valid in the cortical plate and the intermediate zone. Lastly, CO exposed slice co-treated with IBMX had significantly more cells with branches compared to slices treated with CO alone (20.5 ± 1.5 vs. $12.9 \pm 1.5\%$; $p < 0.05$; Figure 6).

Discussion

Interneurons migrate from the GE into the cortical plate

Interneurons that reside in the neocortex arise from the neuroepithelial cell layer of the medial ganglionic eminence in the ventral subpallium (Metin *et al.*, 2006). These cells rely on a number of intrinsic and extrinsic cues to initiate and grow a leading process. The extension of the leading process is one of the first steps in neuronal locomotion; it explores the extracellular environment and responds to repulsive and attractive cues (Marin *et al.*, 2006, Ayala *et al.*, 2007). Process extension allows the migrating cell to “path-find” its route into the cortical plate and to reside in its destined layer. Disruption of this migratory process by an environmental insult can result in permanent displaced interneurons in the cerebral cortex (Poluch *et al.*, 2008). In a previous study, we noted that prenatal exposure to low level CO resulted in an altered distribution of neocortical interneurons. This effect persists into adulthood and caused deficits in behavior associated with sensorimotor gating and pain processing (unpublished data). Based on observations of the effects of prenatal CO exposure, this study furthers understanding of the mechanism mediating CO signaling in organotypic slices.

The CO/NO-cGMP pathway is important for neuronal migration

The major effect of CO is the ability to produce hypoxia by binding to hemoglobin forming carboxyhemoglobin and preventing O₂ delivery to tissues. The developing fetus is particularly vulnerable to insult, as CO readily crosses the placenta and binds to fetal hemoglobin with a higher affinity than adult hemoglobin (Longo, 1970). During chronic exposure, the gas may accumulate in

the tissues and act as a signaling molecule. CO and nitric oxide NO participate in many similar signaling pathways. NO is important for cellular processes involved in development and neuronal migration, particularly through the formation of the second messenger cGMP. The role of NO-mediated production of cGMP through the activation of soluble guanylate cyclase in neuronal migration has been studied in insect models of development. In the hemimetabolous grasshopper (*Locusta migratoria*), Haase and Bicker showed that NO-induced production of cGMP is an essential component of neuronal migration. Neurons fated for the midgut plexus exhibited inducible cGMP immunoreactivity during the migratory phase of development. Furthermore, pharmacological inhibition of endogenous nitric oxide synthase (NOS), soluble guanylate cyclase (sGC), and cGMP-dependent kinase (cGK) each significantly decreased neuronal migration (Haase and Bicker, 2003). Endogenous CO is produced by heme oxygenase (HO) during the metabolism of heme (Boehning *et al.*, 2003), and can signal comparably to NO via the cGMP/cGK cascade. In the grasshopper embryo, the enteric neurons express HO while migrating to the midgut, suggesting CO may also play a critical role in migration (Bicker, 2007). In a model of human neuronal migration, Tegenge and Bicker showed that NO and cGMP positively induce neuronal migration (Tegenge and Bicker, 2009). Other studies have examined the role of NO-induced cGMP production in neuronal migration and development. Ding *et al.* described the developmental expression profile of soluble guanylate cyclase in the mouse, which parallels the period of neuronal migration in the developing brain (Ding *et al.*, 2005). Furthermore, Demyanenko *et al.* showed that knock-out mice with the gene for sGC deleted had abnormal neocortical development consistent with deficiencies in neuronal migration (Demyanenko *et*

al., 2005). Another experimental study used a model of fetal alcohol syndrome in mice, and found that ethanol induced aberrant neuronal migration was reversed by either increasing cGMP levels or decreasing cAMP levels (Kumada *et al.*, 2006). In the mouse embryo, tangentially migrating neurons from the GE naturally produce cGMP earlier than neurons situated in the cortex, and also produce cGMP in response to exogenous NO treatment (Currie *et al.*, 2006). The evidence that cGMP is critical for neuronal migration suggests that alterations of cGMP levels, such as that induced by exogenous CO poisoning, may dramatically affect neuronal migration and neocortical development.

Based on previous studies demonstrating the effects of NO on neuronal migration, we hypothesized that CO could affect migration by affecting the production of cGMP. CO acts as an agonist at sGC similar to NO, and can result in the formation of cGMP (Verma *et al.*, 1993). Our expectation was that CO exposure would result in an increase in cGMP. However, we were surprised to find that chronic exposure *in utero* resulted in a decrease in cGMP in pups at birth (Figure 2). Upon further investigation, we found that although CO activates sGC with a similar mechanism to NO, the affinity for the enzyme is 50-100 times lower (Derbyshire and Marletta, 2009). Therefore, CO acts as a partial agonist to the enzyme during chronic exposure, thereby inhibiting the formation of cGMP by endogenous NO.

Decreased cGMP leads to a decrease in the activation of cGMP dependent kinases, which phosphorylate a number of proteins involved in neuronal migration such as the actin associate complex Ena/VASP. Under normal conditions, phosphorylation of Ena/VASP leads to this protein complex binding to actin, and thereby prevents the binding of other capping proteins. This

results in the continued polymerization of actin, a critical element in neuronal migration (Harbeck *et al.*, 2000). To illustrate the importance of Ena/VASP in neuronal migration and development, a previous study disabled Ena/VASP function through retroviral introduction of FP4-MITO, which binds Ena/VASP to mitochondria. This resulted in a shift of layer 5 pyramidal neurons to upper layers 2 & 3 of the cortex. The authors attributed this effect to either a delayed emergence from the VZ or a decreased rate in migration. Therefore, pyramidal cells normally destined for layer 5 may emerge later and migrate with a cohort of layer 2/3 neurons (Goh *et al.*, 2002). In Figure 2, we show that chronic exposure to CO results in a decrease in phospho-VASP. These decreased levels may have effects on neuronal migration, specifically interneurons migrating from the GE. To further investigate this mechanism, we used an organotypic culture model and studied interneurons migrating from the GE to the neocortex.

CO impaired migration of interneurons into the cortical plate

To study the direct effects of CO as a signaling molecule, we grew embryonic organotypic cultures in either 0 or 50 ppm CO. Interneurons migrating from the ganglionic eminences are a population of migrating cells particularly vulnerable to insult based on their long, tangential path to the cerebral cortex (Wonders and Anderson, 2005, Wonders and Anderson, 2006). Previous work in our lab has shown this population uniquely susceptible to environmental insults (Poluch *et al.*, 2008). Our earlier article studying the effects of CO on cortical organization also indicated a vulnerability of GABAergic cells, therefore we further investigated this population for our study.

Under normal conditions, cells that originated from the GE migrated tangentially into the cortical plate. CO treatment resulted in a nearly 4-fold decrease in cells reaching the cortical plate (Figure 4). Furthermore, the leading processes of treated cells were significantly shorter as seen in Figure 5, suggesting that CO treatment affected cytoskeletal components.

Migration defects caused by CO are reversed by IBMX

The last goal of this study was to determine if CO impaired migration via cGMP dependent pathways. If CO acts as a partial agonist to sGC, then it may be possible to maintain the cGMP produced from endogenous NO by inhibiting its breakdown. To test this idea we grew organotypic slices in both control and CO environments in the presence of the phosphodiesterase inhibitor IBMX. Control slices grown in IBMX demonstrated a trend of decreased migration in cells reaching the cortical plate, however these effects were not statistically significant. Since IBMX is a non-specific phosphodiesterase inhibitor, these effects were likely due to increased cAMP. In CO treated slices, IBMX treatment resulted in a greater than two-fold recovery of neuronal migration to the cortical plate as shown in Figure 4. IBMX treatment also caused a complete recovery in the length of leading processes in CO treated slices (Figure 5), implicating changes in cGMP levels as critical components to the cytoskeletal architecture involved in cellular migration. These data further support that CO impairs migration of cells via the inhibition of cGMP production.

CO directly affects migrating interneurons as a signaling molecule

The results of this study have major implications in understanding CO toxicity, particularly in chronic low levels. CO exposure can occur from a variety of sources including vehicle exhaust, poorly ventilated heating sources, and indoor charcoal cooking. The most common source of low level CO is through cigarette smoke. The prevalence of maternal smoking remains surprisingly high in the US, with about 1 in 5 women continuing to smoke through pregnancy (Phares *et al.*, 2004). This chronic exposure may result in an increased cellular partial pressure of CO and have effects on NO-mediated signaling pathways. Although CO can activate soluble guanylate cyclase with a similar mechanism as NO, the activation is about 50-100 fold less than NO (Derbyshire and Marletta, 2009). CO thereby acts as a partial agonist at the enzyme site, ultimately resulting in a decrease in the normal production of cGMP by endogenous NO. Here we demonstrate that chronic CO exposure results in a decrease in cGMP and phospho-VASP. Furthermore we show that CO can act as a signaling molecule that impairs neuronal migration most likely via effects on cytoskeletal components such as VASP. This effect is rescued with the phosphodiesterase inhibitor IBMX, which caused an improvement in migration and recovery in the length of leading processes of migrating neurons. These data provide further evidence of the role of CO as a signaling molecule, similar to NO, and further illustrate the toxic effect of chronic low level CO exposure on brain development.

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Figure 1. Analysis of organotypic slices

Images of migrating neurons from the GE were analyzed using ImageJ. Arrows were drawn from the cell body to the tip of the leading process. Angles relative to the pia were measured. A cell oriented $0 \pm 15^\circ$ was considered radial. All others were considered tangential. The length of the leading process was measure from the cell body to the tip of the leading edge.

Figure 1. Analysis of organotypic slices

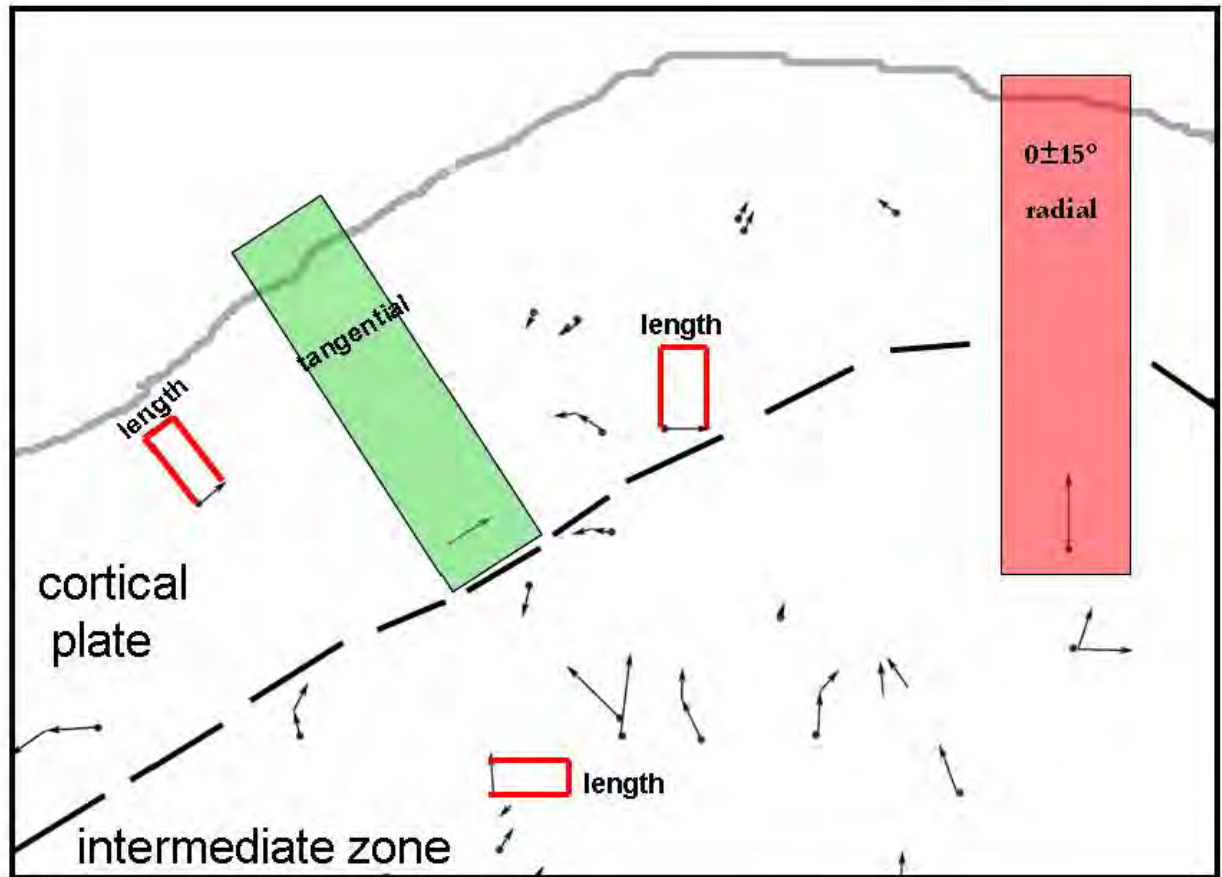


Figure 2. Prenatal CO exposure results in a decrease in cGMP and phosphorylated VASP

Pregnant dams were exposed to 0 or 150 ppm CO from E7 until birth (a) indicates cGMP levels in treated vs. control mice. CO treated pups had significantly reduced cGMP levels compared to controls (0.4 ± 0.1 vs. 0.85 ± 0.153 pg/mL; $n=5$, $p < 0.05$, Student's t-test). (b) shows a western blot of both phospho-VASP (red) and VASP (green). (c) is a bar graph of the quantification of the western blot. Signal intensity from phospho-VASP band was divided by the intensity of VASP band. The values are expressed in integrated optical density units based on those ratios. CO treated pups also had significantly reduced phospho-VASP/VASP ratios compared to controls (1.1 ± 0.3 vs. 1.8 ± 0.1 ; $n=5$, $p < 0.05$, Student's t-test).

Figure 2. Prenatal CO exposure results in a decrease in cGMP and phosphorylated VASP

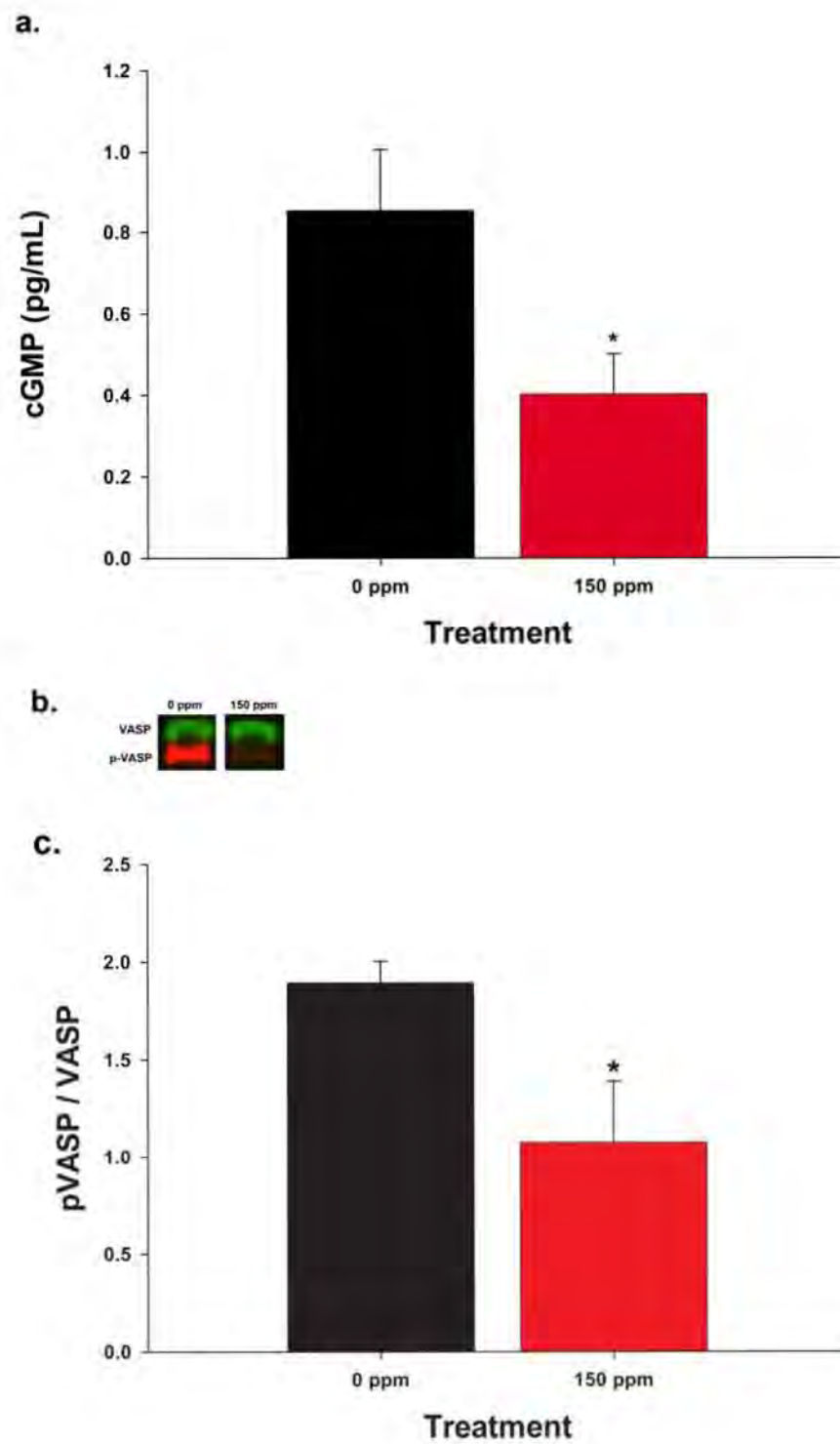


Figure 3. Migrating interneurons were labeled with Dil in E15 organotypic slices.

(a) shows an example of an organotypic slice with Dil labeled cells leaving the ganglionic eminence. Slices were grown for 3 days in either 0 or 50 ppm CO incubators. (b) is a higher power view of the boxed in region, while (c) is a higher power view of migrating neurons. These cells exhibit a distinct leading process, which can have multiple branches that explore the extracellular environment.

Figure 3. Migrating interneurons were labeled with Dil in E15 organotypic slices.

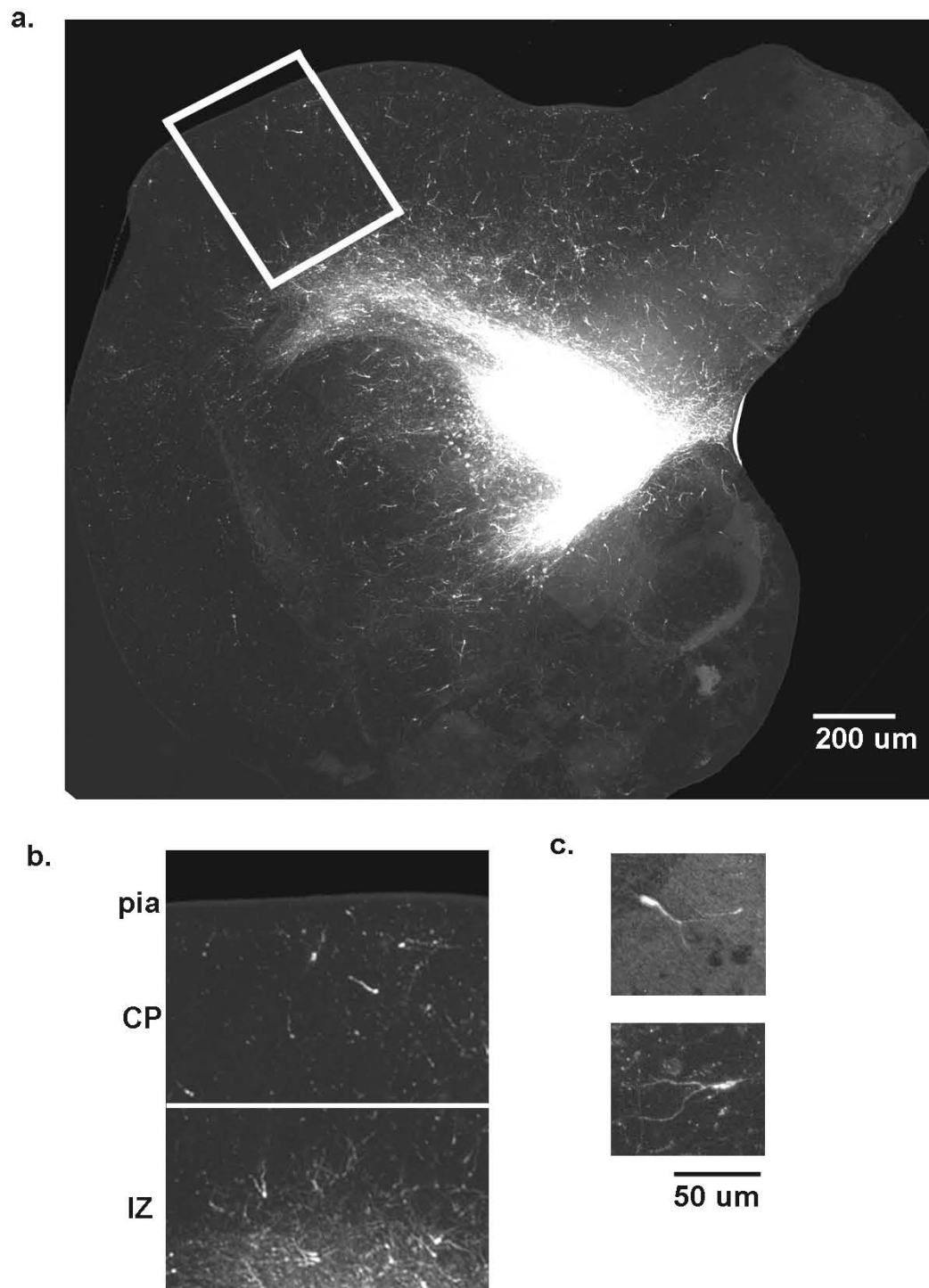


Figure 4. The migration of CO treated cells is impaired. This effect is rescued with IBMX.

Organotypic slices were grown in 0 or 50 ppm CO. The GE was labeled with Dil and the disposition of cells determined after 3 days *in vitro*. In control slices, $53.9 \pm 7.1\%$ of cells reach the cortical plate (CP). Treated slices had a significantly lower percentage of cells reach the CP ($13.8 \pm 3.5\%$). This effect was significantly reversed by the phosphodiesterase inhibitor IBMX (50 μM), with $32.3 \pm 3.5\%$ reaching the cortical plate. (n=5 slices/group; $p < 0.05$, Two-way ANOVA followed by Holm-Sidak post hoc test).

Figure 4. The migration of CO treated cells is impaired. This effect is rescued with IBMX.

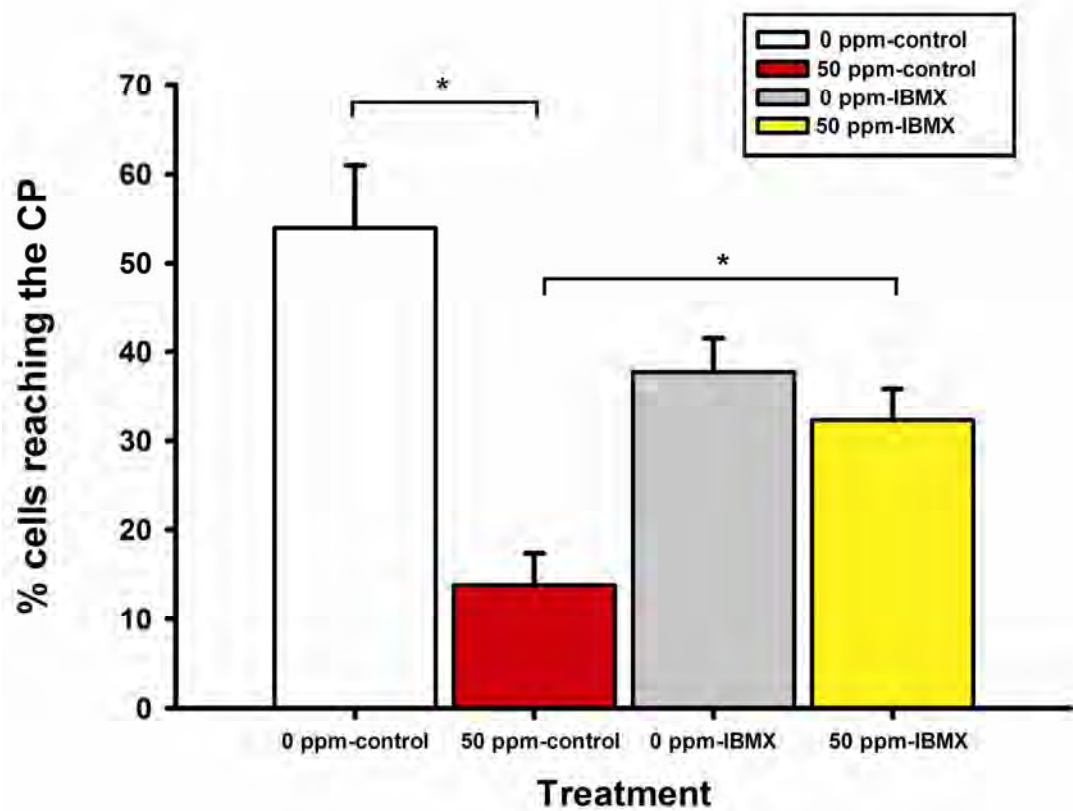


Figure 5. CO exposure results in shorter leading process of migrating cells.

This effect is reversed by IBMX.

Organotypic slices with the GE labeled with Dil were grown in 0 or 50 ppm CO. After 3 days in culture, the length of the leading process of migrating interneurons was measured. In control slices, migrating interneurons had an average leading process length of $78.4 \pm 0.7 \mu\text{m}$ throughout the developing cortex. This was also valid in the cortical plate (CP) and intermediate zone (IZ). In slices treated with 50 ppm CO, cells had significantly shorter leading processes ($64.3 \pm 1.3 \mu\text{m}$). This effect was completely reversed by the phosphodiesterase inhibitor IBMX (50 μM). (n=5 slices/group; $p < 0.05$, Two-way ANOVA followed by Holm-Sidak post hoc test).

Figure 5. CO exposure results in shorter leading process of migrating cells.

This effect is reversed by IBMX

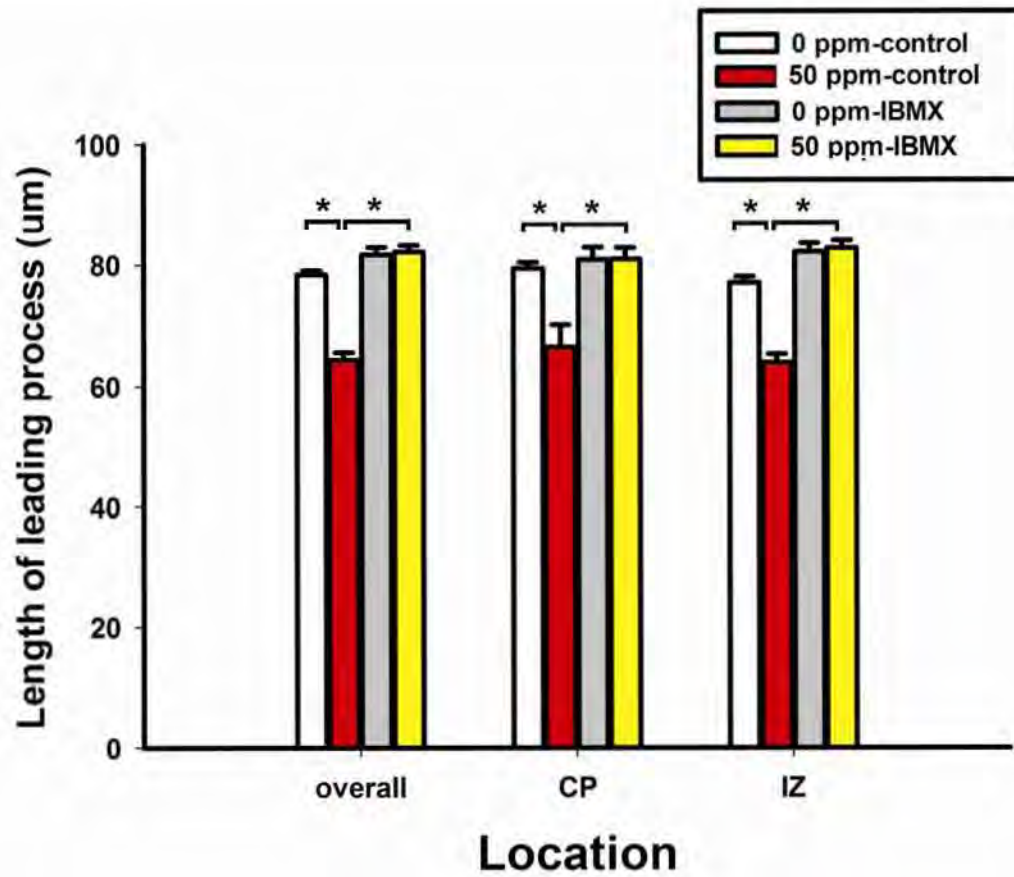


Figure 6. CO treated slices exhibit greater branching when treated with IBMX

E15 organotypic slices with the GE labeled with Dil were grown in 0 or 50 ppm CO environments for 3 days. Cells were grouped as having no branches, one branch, or multiple branches either overall, in the cortical plate (CP), or the intermediate zone (IZ). CO treated slices co-treated with IBMX had significantly more cell exhibiting branches compared to slices treated with CO alone (20.5 ± 1.5 vs. $12.9 \pm 1.5\%$; $n=5$ slices/group; $p < 0.05$, Two-way ANOVA followed by Holm-Sidak post hoc test).

Figure 6. CO treated slices exhibit greater branching when treated with IBMX

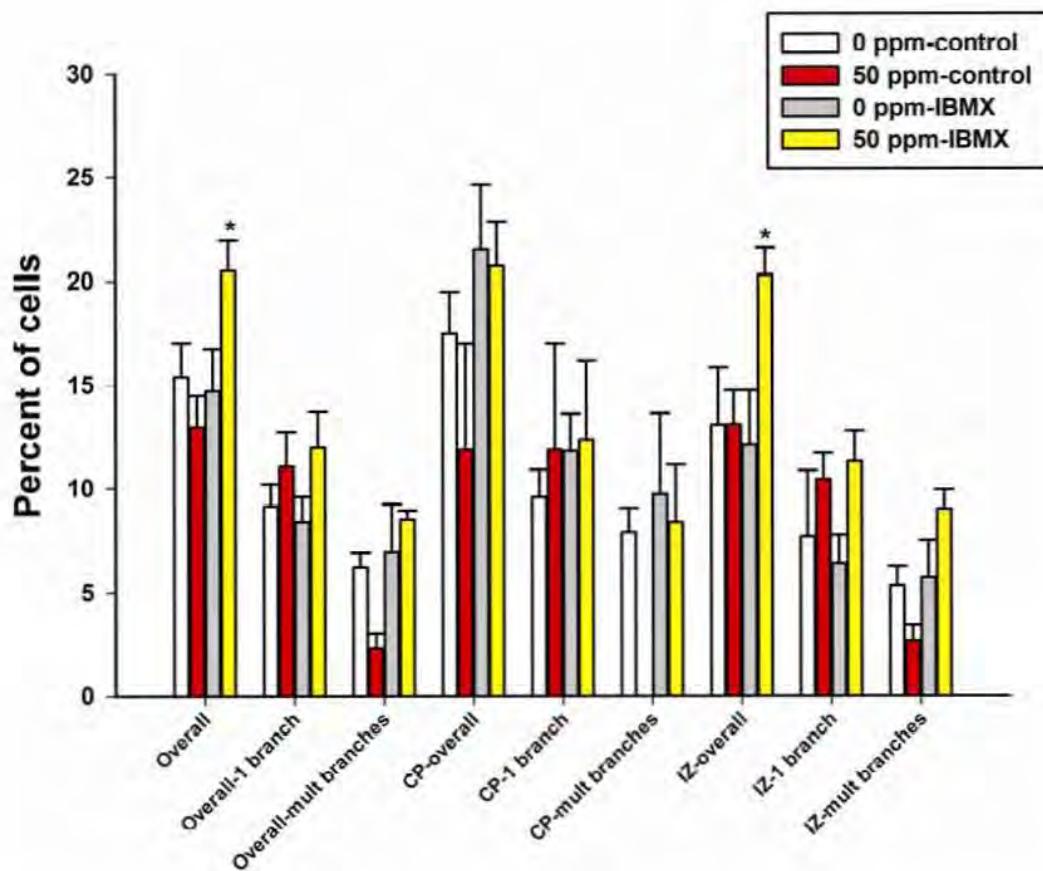
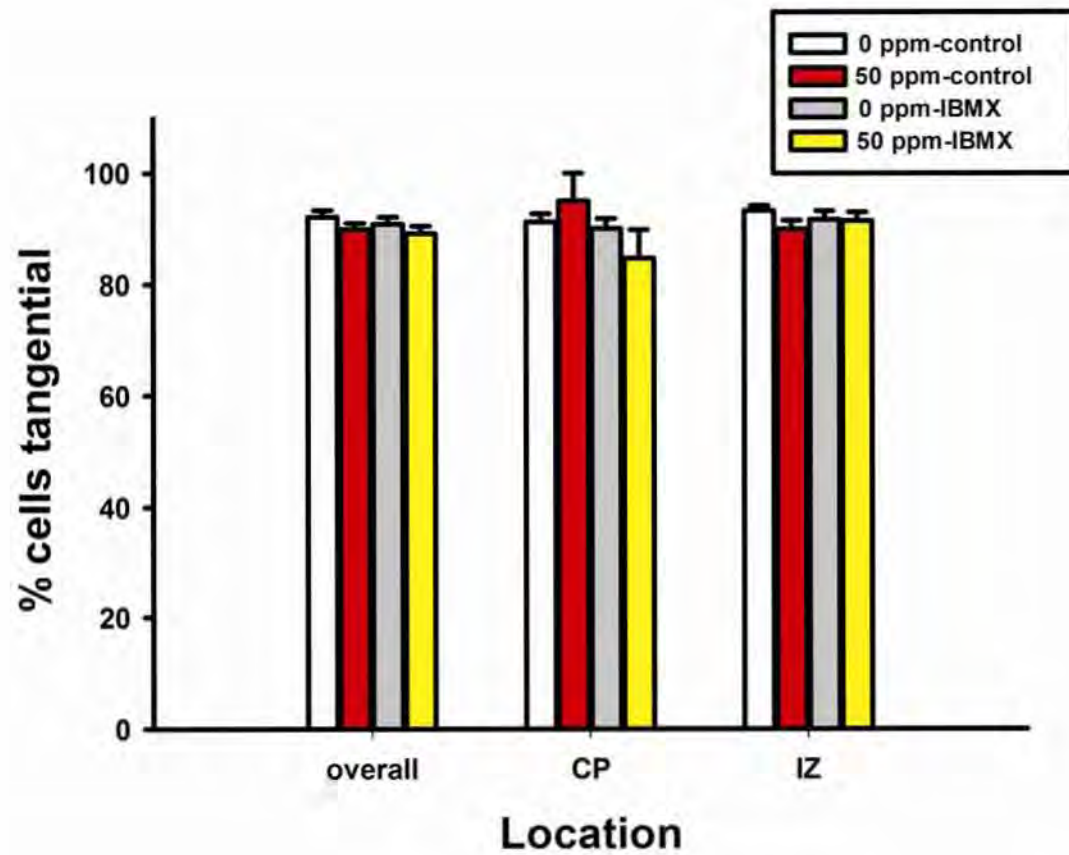


Figure 7. CO does not affect the orientation of migrating interneurons.

E15 organotypic slices with the GE labeled with Dil were grown in 0 or 50 ppm CO environments for 3 days. The orientation of leading processes determined relative to the pia. Cells oriented $0 \pm 15^\circ$ or $180 \pm 15^\circ$ were considered radial, all other considered tangential. In all treatment conditions, over 90% of the cells were tangentially oriented. This was valid in the cortical plate (CP) as well as the intermediate zone (IZ).

Figure 7. CO does not affect the orientation of migrating interneurons.



CHAPTER 5

DISCUSSION

In spite of increasing education in prenatal care, maternal smoking remains a significant threat to neonatal health, with 1 in 5 women continuing to smoke during pregnancy in the US (Phares *et al.*, 2004). Maternal smoking associates with an increased risk for many neurological diseases including microcephaly (Krauss *et al.*, 2003), autism (Hultman *et al.*, 2002), and ADHD (Milberger *et al.*, 1996, Milberger *et al.*, 1998). The effects of maternal smoking are generally attributed to the two major components of cigarette smoke: nicotine and carbon monoxide. While the effects of nicotine are frequently studied and the effects of acute CO poisoning somewhat understood, the effects of chronic low level CO on brain development remains a significant gap in the scientific literature. In this discussion, I highlight the results of our project in the context of prenatal CO altering the structure and function of the cerebral cortex by inhibiting the migration of interneurons in the developing brain. I also highlight alternative mechanisms potentially mediating these effects and discuss future avenues of potential investigation.

PRENATAL CO AFFECTS THE STRUCTURE OF THE CEREBRAL CORTEX

The first specific aim of this study determined if prenatal CO exposure altered the laminar fate of neurons. Our strategy was to inject BrdU at specific dates (E12 or E16) and map the disposition of cells born on that date. This technique is a reliable method to label cells early in development and track their position in the adult cortex (Kriss and Revesz, 1962, Kriegstein, 2005). We noted

a significant alteration in the distribution of BrdU⁺ cells throughout the neocortex. Both early and later born cells were differentially affected, with increased proportions of cells displaced toward the middle layers of the cortex (Figure 1 & Chapter 3, Figure 1). In this model of lineage tracing, the most likely cause of these displaced BrdU⁺ neurons was altered neuronal migration. To date, there are few studies of the effect of CO on neuronal migration and no experiments of this effect in mammalian systems.

The next sets of experiments determined the phenotype of these displaced cells. We confirmed that the majority of BrdU⁺ cells were neuronal by using immunohistochemical co-localization for the neuronal marker NeuN. In accordance with other studies we found that over 70% of BrdU⁺ cells were neuronal (Palmer *et al.*, 2000).

What was the neuronal phenotype of these displaced neurons?

There exist two major populations of neurons in the cerebral cortex. Projection neurons are excitatory in action and use glutamate as their neurotransmitter; interneurons are inhibitory and use GABA. Using the marker MAP2, we showed that the projection neuron distributions in the cortical layers of CO treated mice were similar to control distributions. Furthermore, when colocalized with BrdU administered either early or late during corticogenesis, the distributions of MAP2⁺ neurons in CO treated and normal were similar. This suggested that the displaced cells we noted earlier were not projection neurons. To study the distribution of interneurons, we used immunoreactivity against GAD 65/67, the enzyme responsible for synthesizing GABA. We first noted that the overall interneuron distribution was significantly different in treated mice

compared to controls. Furthermore, the distribution patterns of early and later born interneurons in CO treated versus normal cortex were significantly different, with patterns reflecting that of the overall BrdU⁺ neuronal distribution curves. We also examined two subpopulations of interneurons: parvalbumin⁺ (PV⁺) and calretinin⁺ (CR⁺). PV expressing interneurons arising from the MGE are a unique population of interneurons based on morphology and activity. They reside predominantly in middle to deeper layers of the cortex, have unique basket-like morphology, and are generally display fast-spiking firing (Kawaguchi and Kondo, 2002). CR expressing interneurons arise from the caudal ganglionic eminence (CGE) (Xu *et al.*, 2003). Their migration pattern is unique compared to other cells populating the neocortex in that they migrate in an “outside-in” pattern, through the upper layers to their target layer (Rymar and Sadikot, 2007). They exhibit either a bipolar or multipolar morphology with vertical dendritic arbors spanning the upper layers 2 & 3 of the neocortex (Jacobowitz and Winsky, 1991). In contrast to PV⁺ interneurons, CR⁺ cells are not fast spiking, but heterogeneous in their firing patterns (Kawaguchi and Kubota, 1997). The data given in Chapter 3, Figures 6 & 7 show that mice exposed to CO *in utero* had altered distributions of both PV⁺ and CR⁺ neurons. The proportion of PV⁺ interneurons were significantly reduced in deeper layers and concomitantly increased in upper layers. In contrast, CR⁺ cells were proportionally reduced in upper layers of the cortex.

These data have significant physiological implications, since a change in interneuron distribution may have profound impact on cortical function. In general, an overall level of excitatory/inhibitory balance is maintained in the neocortex. Although interneurons only represent about 20-30% of neurons, they are responsible for modulating the complex excitatory activity of the cortex.

Subtle changes in this balance could underlie spontaneous evoked potentials that give rise to seizures (Badawy *et al.*, 2007) or affect executive function behaviors requiring effective cognitive inhibition (Figure 2). For example, in post-mortem analyses of the brains of schizophrenic patients, researchers revealed alterations in interneuron distribution as an explanation of signs and symptoms associated with the disease (Raghanti *et al.*, 2010). Autistic patients have altered numbers of PV expressing interneurons (Casanova *et al.*, 2003). The results of the first specific aim, therefore, show changes in the structure of the cerebral cortex after prenatal CO exposure that parallel aspects of neurological diseases, which are also associated with maternal smoking.

Alternative outcomes and future experiments

The results of this portion of the project provide evidence that prenatal CO affects the laminar fate of interneurons migrating into the neocortex. However, we only looked at two distinct times of development, E12 and E16. It is possible that neurons fated for other layers are also affected, which would be more precisely revealed by BrdU injections on other embryonic days. Preplate neurons are sensitive to hypoxic-ischemic injury (McQuillen *et al.*, 2003, McQuillen and Ferriero, 2004); therefore we may see altered migration patterns in very early born cells. Since preplate cells are a transient cell population, we may only see distinctions in younger animals, as this cell layer disappears after the first week of development in the mouse (McQuillen *et al.*, 2003). On the other hand, since subplate cells are reported to influence incoming cortical neurons, aberrations may also be present in later born populations of cells as well. Since deficiencies in righting reflexes have been seen in previous studies (Singh, 1986)

and spastic motor deficits observed in neonatal hypoxic-ischemia (McQuillen *et al.*, 2003) these symptoms may be reflected in altered distributions of layer 5 pyramidal neurons, which would be consistent with upper motor neuron damage, and be detected with E13 or E14 BrdU injections. If layers 2-4 are affected, we may find altered thalamocortical input to layer 4 resulting in abnormal intercolumnar responses to sensory stimuli. In our laboratory's ferret model of cortical dysplasia, disruption of layer 4 development results in an altered pattern of responses to thalamic stimulation, suggesting an abnormal processing of sensory stimuli that may result in perceptual changes (McLaughlin and Juliano, 2005). Alterations in these layers would be observed after E14 or E15 BrdU injections. Finally, CO administration may delay proliferation of cells destined for the cortex, which could be detected in E18 injected mice. If there was a delay in the timing of leaving the cell cycle, we might detect a greater proportion of BrdU+ cells in CO treated mice compared to controls. This effect could be global, in that all cells may emerge from the VZ or GE later in development than normal due to effects of CO.

To further understand the specific layers impacted by this, we could also have used layer specific markers. Do the displaced neurons take on the phenotype of the surrounding neurons in that layer? Are neural progenitor cells multi-potent, or is a neuron programmed for a specific layer as soon as it exits the cell cycle? Since we found that interneurons were specifically affected, it is possible that their migration into the cortex was slower than normal. Since tangentially migrating cells rely on extracellular cues to guide them to their location, a transient delay in reaching the cortical plate may have caused aberrant responses to the appropriate cue. For example, if cell A is migrating to

cell B, it relies on cell B to produce an attractant molecule along a gradient. If this gradient is established at a specific time, such as E15, and cell A is slower to reach the cortical plate, then cell A may have missed the critical attractant cue from cell B. This would result in cell A to be displaced in the cortex.

In conclusion, the results from these experiments demonstrate that prenatal CO results in alterations in the architecture of the cerebral cortex. The next critical question to be answered was whether these subtle changes in structure resulted in a change in function.

PRENATAL CO IMPAIRS THE FUNCTION OF THE CEREBRAL CORTEX

Based on the results from the previous experiments, the next question we addressed was whether prenatal CO exposure had functional consequences. Our design strategy was based on established behavioral consequences of CO as well as neuropathological associations with maternal smoking.

Prenatal exposure to CO disrupts long-term potentiation in the hippocampus, suggesting deficiencies in learning and memory (Mereu *et al.*, 2000). At low concentrations (75-150 ppm) CO induced a variety of neurobehavioral abnormalities in rat offspring including changes in activity levels through preweaning, delayed development of homing behavior and negative geotaxis, altered ontogeny of emotional responsiveness, and permanent cognitive deficits (Fechter and Annau, 1977, 1980, Mactutus and Fechter, 1984, 1985, Di Giovanni *et al.*, 1993). Cagiano *et al.* demonstrated in male rats that low prenatal CO concentrations elicited changes in mesolimbic dopaminergic function and impaired sexual behavior (Cagiano *et al.*, 1998). Storm and Fechter found changes in catecholamines in the cerebellum after *in utero* CO exposure

and linked these with deficiencies in motor test performance and assessments of learning and memory (Storm and Fechter, 1985).

Maternal smoking associates with a number of neurological consequences such as autism and attention deficit disorder (Agrawal *et al.*, 2010, Milberger *et al.*, 1996, Milberger *et al.*, 1998, Larsson *et al.*, 2005). We, and others, have now shown that maternal smoking also correlates with cortical dysplasias (Krauss *et al.*, 2003), which in turn are linked with schizophrenia and epilepsy (Reiss-Zimmermann *et al.*, 2010, Reynolds and Beasley, 2001). Schizophrenic patients have a unique behavioral spectrum with behaviors that can be modeled in laboratory animals. One test that is conserved across many mammalian species from rodent to human is the pre-pulse inhibition test. This test measures the subject's reflex to an acoustic startle (ASR), and the ability to inhibit the ASR when presented with a lower intensity sound 20-100 ms preceding the acoustic startle. This effect is referred to as a sensorimotor gating ability and is a complex network of pathways involving brainstem, limbic, and neocortical inputs (Li *et al.*, 2009), (Hoffman and Ison, 1980). Both schizophrenic and ADHD patients have diminished or absent pre-pulse inhibition (Braff *et al.*, 1978, Kumari *et al.*, 2000, Hawk *et al.*, 2003). Autistic patients exhibit patterns of repetitive behavior, altered emotional responsiveness, and impaired social interaction. They also exhibit altered pain thresholds, which is thought to be mediated by a change in central mediated pain processing (Sahley and Panksepp, 1987). These clinical signs of impaired sensorimotor gating and altered pain processing can be modeled in rodents. In this study, we showed that indeed mice exposed to CO *in utero* do exhibit such clinical signs based on results from the open field test, the pre-pulse inhibition test, and the hot plate test.

Based on other models of cortical dysplasia and of altered interneuron function, we expected that prenatal exposure to CO would affect overall activity in treated mice compared to controls. In a model of cortical dysplasia employing the antimitotic compound methyloxymethanol (MAM), other researchers reported hyperactivity in juvenile rats exposed to MAM during gestation (Nishida *et al.*, 1992, Fiore *et al.*, 2000). In another study of interneuron function, Levav *et al.* examined the effects of altering GABA content early in development using the antiepileptic drug vigabatrin (Levav *et al.*, 2008). Exposure to vigabatrin during early postnatal development was shown to increase activity in P14 mice, but decreased overall activity in adults, when assessed with the open field test (Levav *et al.*, 2008). These data suggested that we may have also expected to see differences in locomotor activity in mice exposed to prenatal CO.

However, treated mice did not possess differences in locomotor activity compared to controls in 12 parameters of activity. This indicated that no gross motor deficits existed between groups and the CO treated animals had relatively normal levels of activity, anxiety and exploratory behavior. For more sensitive domains of fine motor function, we could test mice on the rotarod for balance and coordination or the footprint pattern test for ataxia and abnormalities of gait. The open field test was the most logical starting point to study motor deficits in our model.

Since maternal smoking is associated with an increased incidence of ADHD (Milberger *et al.*, 1998), cortical dysplasia associates with schizophrenia (Reynolds and Beasley, 2001), and these disorders are linked with impairment of sensorimotor gating, we expected a similar decrease in pre-pulse inhibition (PPI) in mice exposed to prenatal CO (Hawk *et al.*, 2003, Moore *et al.*, 2006, Swerdlow

et al., 2008). We tested the ASR to 110 dB in mice exposed to CO compared to controls, as well as the PPI when the startle stimulus was preceded by 68, 79, or 90 dB. In all three cases, CO treated mice lacked pre-pulse inhibition compared to controls, who exhibited approximately 80% ASR when presented with a pre-pulse. We were concerned that impaired auditory processing in CO exposed mice may have confounded our results, since previous studies in rats showed that perinatal exposure to low levels (100 ppm or less) of CO resulted in decreased development of the inferior colliculus and impaired auditory processing (Stockard-Sullivan *et al.*, 2003, Webber *et al.*, 2003). However since baseline acoustic startle reflexes were similar in CO treated mice compared to controls, auditory processing was not likely impaired. These results clearly indicate a disruption of sensorimotor gating. To confirm these results in future experiments, an alternative cognitive test is the 5-choice serial reaction test, which challenges selective attention in a mouse as it must monitor up to nine holes for a blink of light followed by a food reward. The PPI was an ideal assessment of sensorimotor gating that had been well characterized in mice and indicated in a number of pathologies that have similar outcomes associated with prenatal CO exposure.

The last behavioral paradigm we tested was central mediated nociception using the hot plate test. CO exposed mice had altered interneuron distribution, which may indicate altered supraspinal mediated processing. Previous studies have shown a critical role of GABAergic transmission in supraspinal mediated pain processing. In a mouse knockout model, researchers found that mice lacking GAD-65 demonstrated hyperalgesia (Kubo *et al.*, 2009). In another transgenic mouse model, mice were bred to overexpress the GABA transporter

GAT-1, thereby altering the tonic GABA levels in the synaptic environment. These mice exhibited hyperalgesia to a variety of thermal and chemical nociceptive challenges (Hu *et al.*, 2003). The importance of the cerebral cortex to modulate pain pathways was demonstrated in a study that used focal injections of GABA agonists and antagonists. They showed that inhibitory interneurons play a dual role to produce either analgesia or hyperalgesia, depending on non-specific activation of GABA receptors or specifically GABA_B, respectively (Jasmin *et al.*, 2003). In our study, mice exposed to CO *in utero* had significantly shorter hot plate latencies compared to controls, consistent with previous reports that alterations in GABAergic activity can influence nociception. Further studies to expand on these data could include using alternative techniques to measure nociception aside from thermal stimulation, such as chemical or mechanical stimulation.

Overall, these data show that prenatal exposure to CO produces functional changes in offspring. Future studies can further characterize functional changes in more diverse behavioral paradigms. Another avenue of research in this model would be to examine the functional outcome with electrophysiology. We initiated experiments using field potential recordings throughout specific layers of the cortex. Preliminary data showed a reduction in excitatory activity in layers 2/3, and increase in excitatory activity in layer 5 in treated mice compared to controls. However, the sample size in this group was small (n=1/group), therefore no definitive conclusions can be made.

Since we determined that prenatal CO exposure has functional consequences, the next challenge was determining the mechanism(s) mediating this effect.

CO DIRECTLY IMPAIRS NEURONAL MIGRATION VIA THE NO/cGMP/cGK-II PATHWAY

The third component of this project determined the mechanism mediating CO impairment of neuronal migration. Our strategy for this was to grow organotypic slices in CO and control environments. One confounding variable presented with *in vivo* exposure to CO lies in the effects of hypoxia produced by CO binding with hemoglobin producing carboxyhemoglobin. By using organotypic slices, we were able to examine the direct effects of CO gas on the tissue. We also knew that interneurons were uniquely susceptible to perturbation by CO, therefore specifically targeted the migration of cells arising from the ganglionic eminence.

While our initial observations *in vivo* noted alterations in interneuron migration, we did not know whether CO was a facilitator or an inhibitor of migration. Because we knew that CO can act as a gaseous signaling molecule similar to NO, we hypothesized that CO affected migration by acting through the NO-mediated production of cGMP. The role of NO in neuronal migration has been studied in insect models of development. In the hemimetabolous grasshopper (*Locusta migratoria*), Haase and Bicker showed that NO-induced production of cGMP is an essential component of neuronal migration. Neurons fated for the midgut plexus exhibited inducible cGMP immunoreactivity during the migratory phase of development. Furthermore, pharmacological inhibition of endogenous NOS, sGC, and cGK each significantly decreased neuronal migration (Haase and Bicker, 2003). Endogenous CO is produced by heme oxygenase (HO) during the metabolism of heme (Boehning *et al.*, 2003), and can signal similar to NO via the cGMP/cGK cascade. In the grasshopper embryo, the

enteric neurons express HO while migrating to the midgut, suggesting CO may also play a critical role in migration (Bicker, 2007). In a model of human neuronal migration, Tegenge and Bicker showed that NO and cGMP positively induce neuronal migration (Tegenge and Bicker, 2009). Mice with deletion of the gene for sGC had abnormal neocortical development consistent with deficiencies in neuronal migration (Demyanenko *et al.*, 2005). Another experimental study used a model of fetal alcohol syndrome in mice, and found that the alteration of neuronal migration by ethanol was reversed by either increasing cGMP levels or decreasing cAMP levels (Kumada *et al.*, 2006). In the mouse embryo, tangentially migrating neurons from the GE naturally produce cGMP earlier than neurons situated in the cortex, and also produce cGMP in response to exogenous NO treatment (Currie *et al.*, 2006). The evidence that cGMP is critical for neuronal migration suggests that alterations of cGMP levels, such as that induced by exogenous CO poisoning, may dramatically affect neuronal migration and neocortical development. Based on cGMP being an inducer of migration, changes in levels caused by CO could explain the migration impairment we noted *in vivo*.

Does CO exposure cause cGMP to increase or decrease?

Although CO can activate soluble guanylate cyclase with a similar mechanism as NO, the activation is about 50-100 fold less than NO (Derbyshire and Marletta, 2009). On one hand, CO may positively influence the system as an agonist, and result in an increase in cGMP. If that were the case, we would see an increase in the migration of cells leaving the GE in our organotypic slices. Rescue of this effect could potentially occur by inhibiting the production of cGMP,

or inhibiting its target, cGK. However, these were not the initial observations. In contrast, CO treated slices exhibited significantly reduced migration compared to controls. Pharmacological inhibition of the cGMP mediated pathways exacerbated this effect, resulting mostly in slice death. This forced us to rethink our hypothesis that CO was a positive regulator of migration similar to NO.

The alternative hypothesis is that CO acts as a partial agonist at the enzyme site, ultimately resulting in a downstream decrease in the normal production of cGMP by endogenous NO. Decreased cGMP then leads to a decrease in the activation of cGMP dependent kinases, which phosphorylate a number of proteins involved in neuronal migration such as the actin associated complex Ena/VASP. These were two parameters that could be assayed in the CO exposed mice. We found that mice exposed to CO *in utero* and analyzed at birth (P0) had reduced cGMP and lower levels of phosphorylated VASP.

Under normal conditions, phosphorylation of Ena/VASP leads to it binding to actin, and thereby prevents the binding of other capping proteins. This results in the continued polymerization of actin, a critical element in neuronal migration (Harbeck *et al.*, 2000). To illustrate the importance of Ena/VASP in neuronal migration and development, a previous study disabled Ena/VASP function through retroviral introduction of FP4-MITO, which bound Ena/VASP to mitochondria. This resulted in a shift of layer 5 pyramidal neurons to upper layers 2 & 3 of the cortex (Goh *et al.*, 2002).. The authors attributed this effect to either delayed emergence from the ventricular zone (VZ) or decreased rate in migration. Therefore, pyramidal cells that are normally destined for layer 5 may have emerged later and migrated with a cohort of layer 2/3 neurons. These reports are consistent with our results suggesting that CO reduced the amount of

cGMP and phospho-VASP, which was responsible for the impairment of migration.

To support this hypothesis, we designed experiments using a variety of drugs to increase NO levels, simulate cGMP, or salvage what cGMP was produced endogenously. Treatment with NO donors resulted in significant slice death in the CO treated group. This effect was likely caused by an overwhelming amount of NO available, which may have formed free radicals damaging the tissue and resulting in cell death. Treatment with a synthetic cGMP also failed to rescue the effect of CO. This is likely due to the relatively short half life of the drug used, compared with the duration of CO exposure. Finally, we used the phosphodiesterase inhibitor IBMX to preserve what cGMP was formed. IBMX successfully rescued the migration impairment caused by CO, resulting in a greater than 2.5-fold increase in cells reaching the cortical plate. We hypothesized that CO affected migration by altering VASP phosphorylation, therefore expected to see cytoskeletal alterations in migrating cells. In CO treated cells, the length of the leading processes were significantly shorter compared to controls. This effect was completely rescued by IBMX. Furthermore, CO exposed cells treated with IBMX had significantly more branching compared to CO exposed cells not treated with IBMX. Overall, these results support the idea that CO signaling mediated by cGMP acts to alter cytoskeletal changes required for neuronal migration.

Since we did not note complete recovery of migration, there are likely other potential mechanisms mediating the impairment of migration by CO. One is that these effects are due to inhibition of important energy generating mitochondrial processes by CO (Chance *et al.*, 1970). Cytochrome oxidase

(CcOX) is the heme containing enzyme of the electron transport chain that can bind CO. In myocardial cells, CO exposure causes reduction in CcOX activity by lowering V_{\max} and reduced heme aa₃ content resulting in decreased aerobic ATP production (Iheagwara *et al.*, 2007). In neurons, *in vitro* exposure to high doses of CO (300 – 1000 ppm) produced a loss of mitochondrial membrane potential and initiation of apoptosis (Tofighi *et al.*, 2006). Programmed cell death is an important component of neocortical development. Premature apoptosis could explain differences in the laminar fate of neurons, and could be measured using a TUNEL assay to detect DNA fragmentation. Mitochondrial function could be studied in embryonic brains in CO exposed groups compared to controls by measuring the amount of mitochondria, the electrochemical mitochondrial gradients with tetramethylrhodamine ethyl ester (TMRE), or by using uniformly radiolabeled glucose to measure CO₂ production. This would detect metabolic differences in CO treated brains compared to controls. Since neuronal migration is an extremely metabolically demanding process, alterations in metabolism would likely have an effect.

Overall, these data are exciting in that they provide further evidence of CO acting as a signaling molecule at the tissue level. While most studies attribute the toxic effects of CO to its ability to produce hypoxia, there are also effects at the tissue level that can be rescued pharmacologically. Furthermore, the use of CO-releasing molecules therapeutically as vasodilators similar to nitrates is of increasing clinical interest. This study shows significant side effects of CO as a signaling molecule in migrating neurons. While CO releasing drugs will likely be contraindicated in pregnant women, there remains significant neurogenesis and neuronal migration in the adult brain, particularly in the hippocampus and

olfactory bulb. The effect of chronic, low level CO on the structure and function of these structures in the adult brain remains to be studied.

CONCLUSIONS

In summary, this research shows that prenatal exposure to low level CO, similar to what is seen in a chronic smoker, results in defects in the migration of interneurons populating the cerebral cortex. This also results in functional deficits in offspring that persist into adult, with impairments noted in sensorimotor gating and supraspinal mediated nociception. Lastly, the results indicate that this effect is mediated by CO acting as a signaling molecule as a partial agonist at sGC, a target of NO, resulting in a decrease in cGMP and decrease in its downstream target, phospho-VASP. This effect explains the impairment of migration via cytoskeletal proteins. In addition, neuronal migration is rescued, in large part, by the phosphodiesterase inhibitor IBMX, which would act to preserve cGMP levels.. These data support the hypothesis that chronic low level CO is toxic to the developing neocortex, produces functional deficits consistent with associations with maternal smoking and cortical dysplasias, and that CO acts directly as a signaling molecule to affect neuronal migration.

Figure 1. CO exposure alters the laminar fate of cells in the neocortex.

Pregnant dams were exposed to 0 or 150 ppm CO from E7 until birth. Bromodeoxyuridine (BrdU) was injected at either E12 (early born cells) or E16 (later born cells). This representation from results in Chapter 3 illustrate that mice exposed to CO *in utero* had altered distributions of both early (E12) and later (E16) born cells in somatosensory cortex.

Figure 1. CO exposure alters the laminar fate of cells in the neocortex.

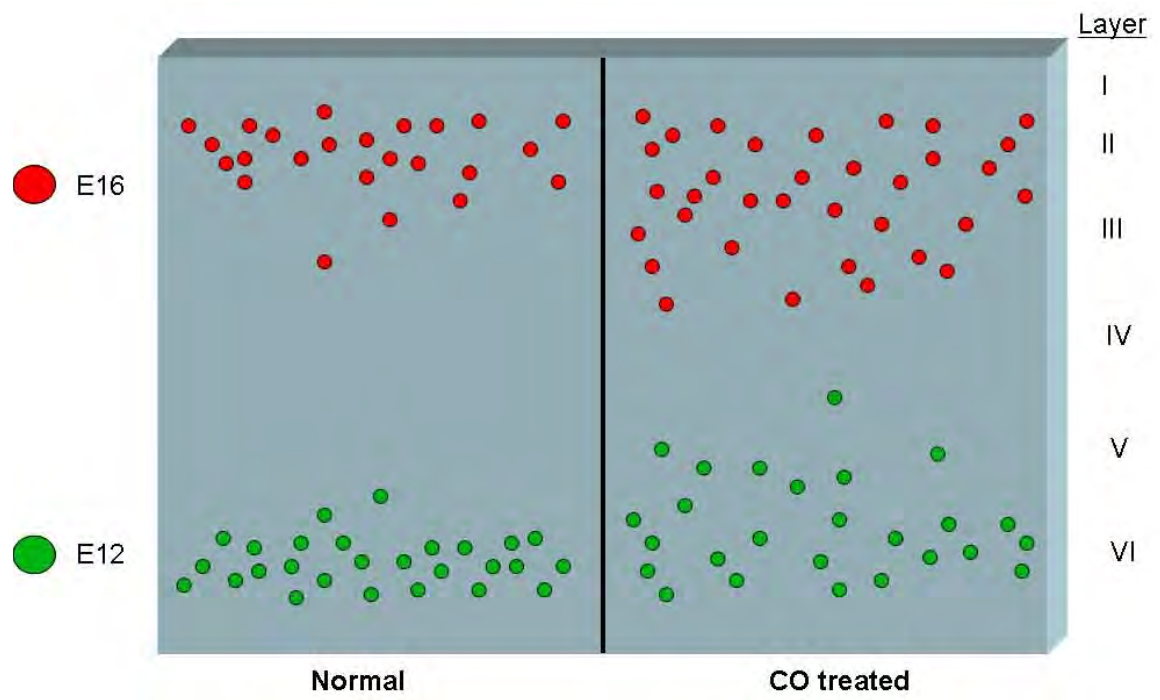


Figure 2. Prenatal CO may alter the excitatory/inhibitory balance in the cortex.

Under normal conditions, there exists a balance of excitation and inhibition in the cerebral cortex. This balance is maintained, in part, by the specific distribution and activity of GABAergic interneurons throughout the cortex. In mice exposed to CO *in utero* (b), there is a significant alteration in the distribution of interneurons throughout the cortex. This may affect the overall excitatory/inhibitory balance of the cortex, and explain some of the functional deficits seen in CO exposed mice.

Figure 2. Prenatal CO may alter the excitatory/inhibitory balance in the cortex.

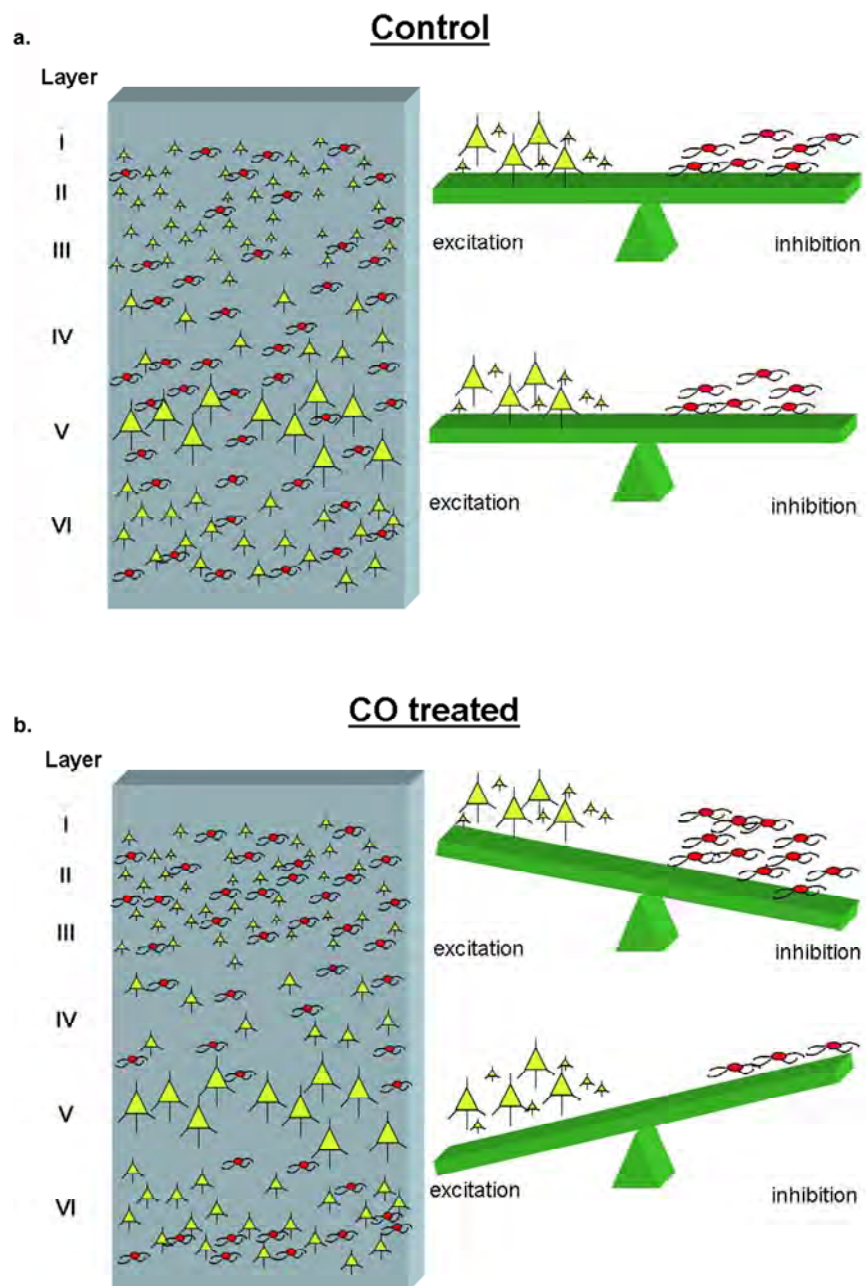
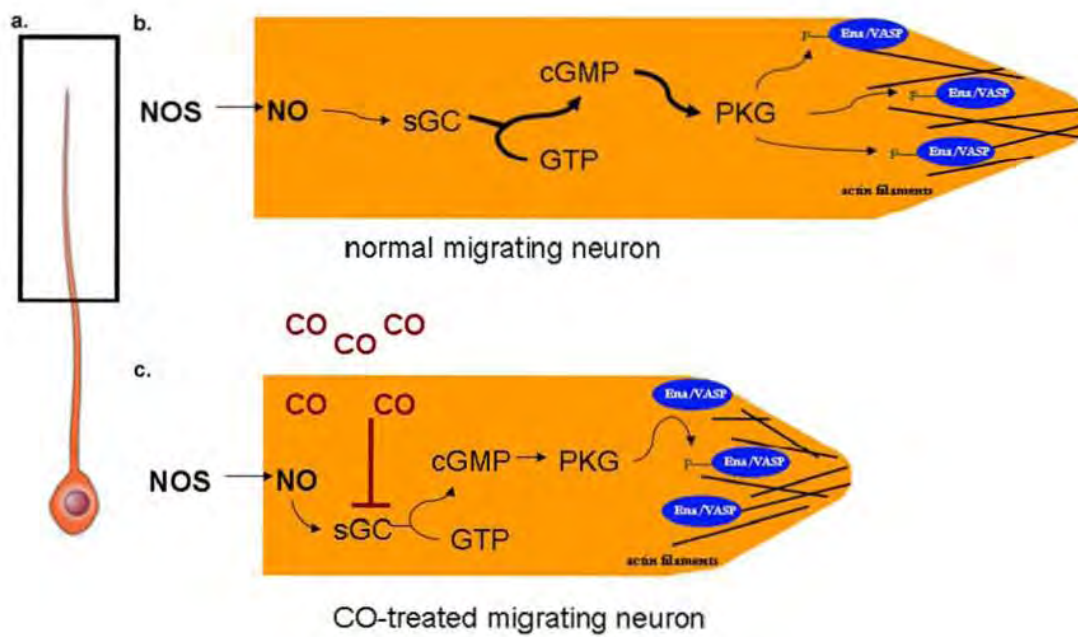


Figure 3. The CO/NO-cGMP-cGK pathway is important for neuronal migration.

A signaling cascade regulating neuronal migration that was affected by CO is the CO/NO-cGMP pathway. (a) This schematic represents the leading process of a migrating neuron. Under normal conditions (b), nitric oxide (NO) is produced by nitric oxide synthase (NOS). NO then activates soluble guanylate cyclase (sGC) to form cyclic guanosine monophosphate (cGMP). cGMP then activates cGMP-dependent kinase (PKG), which phosphorylates the actin associated protein Ena/VASP. Phosphorylation of Ena/VASP results in an increase in actin filament formation and increase in migration. (c) CO acts as a partial agonist to sGC, thereby diminishing the effect of endogenous NO. This resulted in decreased cGMP levels, lower amounts of VASP phosphorylation, and impaired neuronal migration.

Figure 3. The CO/NO-cGMP-cGK pathway is important for neuronal migration.



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